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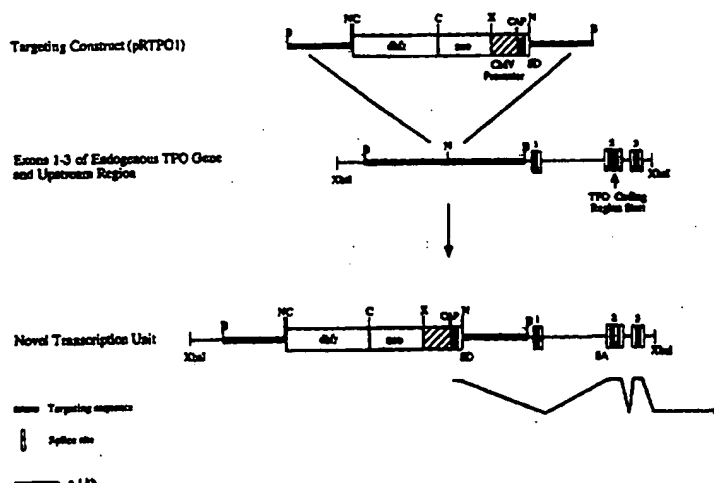
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| (51) International Patent Classification ⁶ : C12N 15/19, 15/22, 15/55, 15/67, 9/16, 5/10, C07K 14/52, 14/565, A61K 31/70, 38/19, 38/21, 38/46, 48/00 | | A1 | (11) International Publication Number: WO 96/29411 (43) International Publication Date: 26 September 1996 (26.09.96) |
| (21) International Application Number: PCT/US96/03377 (22) International Filing Date: 12 March 1996 (12.03.96) (30) Priority Data: 08/406,030 17 March 1995 (17.03.95) US (60) Parent Application or Grant (63) Related by Continuation US 08/406,030 (CIP) Filed on 17 March 1995 (17.03.95) (71) Applicant (for all designated States except US): TRAN- SKARYOTIC THERAPIES, INC. [US/US]; 195 Albany Street, Cambridge, MA 02139 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): TRECO, Douglas, A. [US/US]; 87 Brantwood Road, Arlington, MA 02174 (US). HEARTLEIN, Michael, W. [US/US]; 167 Reed Farm Road, Boxborough, MA 01719 (US). HAUGE, Brian, M. [US/US]; 17 Clifton Avenue, Beverly, MA 01915 (US). SELDEN, Richard, F. [US/US]; 106 Bristol Road, Wellesley, MA 02181 (US). | | (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith, Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. | |

(54) Title: PROTEIN PRODUCTION AND DELIVERY



(57) Abstract

The invention relates to novel human DNA sequences, targeting constructs, and methods for producing novel genes encoding thrombopoietin, DNase I, and β -interferon by homologous recombination. The targeting constructs comprise at least: (a) a targeting sequence; (b) a regulatory sequence; (c) an exon; and (d) a splice-donor site. The targeting constructs, which can undergo homologous recombination with endogenous cellular sequences to generate a novel gene, are introduced into cells to produce homologously recombinant cells. The homologously recombinant cells are then maintained under conditions which will permit transcription of the novel gene and translation of the mRNA produced, resulting in production of either thrombopoietin, DNase I, or β -interferon. The invention further relates to methods of producing pharmaceutically useful preparations containing thrombopoietin, DNase I, or β -interferon from homologously recombinant cells and methods of gene therapy comprising administering homologously recombinant cells producing thrombopoietin, DNase I, or β -interferon to a patient for therapeutic purposes.

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PROTEIN PRODUCTION AND DELIVERY

Background of the Invention

Current approaches to treating disease by administering therapeutic proteins include in vitro production of therapeutic proteins for conventional pharmaceutical delivery (e.g. intravenous, subcutaneous, or intramuscular injection, or by intranasal or intratracheal aerosol administration) and, more recently, gene therapy.

One protein which may be useful in the treatment of platelet disorders is thrombopoietin (TPO). Platelets are small (2-3 microns in diameter) anucleated cells which play an important role in primary hemostasis by adhering to and aggregating at sites of vascular damage. In addition, platelets release factors which are important components of the blood coagulation, inflammation, and wound healing pathways. Patients with very low levels of circulating platelets (thrombocytopenia) exhibit bleeding into superficial sites (e.g. skin, mucous membranes, genitourinary tract, and gastrointestinal tract) as a result of mild trauma, and are at risk for death from catastrophic hemorrhage occurring spontaneously or resulting from trauma. The physiologic role of platelets and the etiology of platelet disorders have been described (cf. *Hematology: Clinical and Laboratory Practice*, Eds. R.L. Bick et al., pp. 1337-1389, Mosby, St. Louis (1993); *Harrison's Principles of Internal Medicine*, Eds. J.D. Wilson et al., 11th Ed., pp. 1500-1505, McGraw Hill, New York, 1991).

Thrombocytopenia may be caused by decreased production of platelets by the bone marrow, increased sequestration of platelets in the spleen, or accelerated platelet destruction. Decreased production of platelets by the bone marrow may result from destruction of hematopoietic precursor cells by irradiation or treatment with cytotoxic agents during therapy for cancer. In addition, alcohol,

-2-

estrogens, and thiazide diuretics can suppress platelet production (drug-induced thrombocytopenia). Furthermore, infiltration of the bone marrow by malignant cells and the disorders congenital amegakaryocytic hypoplasia and throm-
5 bocytopenia with absent radii (TAR syndrome) can result in decreased platelet production.

Increased splenic sequestration of platelets may occur as a result from splenomegaly associated with a variety of conditions, including liver disease, infiltration of the
10 spleen with tumor cells as in myeloproliferative or lymphoproliferative disorders, and Gaucher's disease.

Accelerated platelet destruction and thrombocytopenia may be caused by vasculitis, hemolytic uremic syndrome, disseminated intravascular coagulation, and the presence of
15 intravascular prosthetic devices such as cardiac valves. In addition, certain viral infections, drugs, and autoimmune disorders lead to immunologic thrombocytopenia in which platelets become coated with antibody, immune complexes, or complement and are rapidly cleared from the
20 circulation. A number of drugs can elicit an immune response leading to immunologic thrombocytopenia, including sulfathiazole, novobiocin, para-aminosalicylate, quinidine, quinine, carbamazepine, digitoxin, arsenical drugs, and methyl dopa.

25 Thrombocytopenia is currently treated most readily by transfusion with platelet concentrates, although corticosteroid therapy or plasmapheresis can be effective in immunologic thrombocytopenia. Treatment with platelet concentrates is severely limited by availability of suitable donors and the risk of transmission of blood-borne
30 infectious diseases.

As an alternative to transfusion therapy, platelet deficiencies could be treated with hematopoietic growth factors which promote proliferation and maturation of
35 megakaryocytes, the nucleated progenitor cells from which

-3-

platelets are derived. Recently, cDNA clones were isolated which encode the human, mouse, and dog analogs of a protein purified from aplastic porcine plasma which displays megakaryocytopoietic activity (de Sauvage, F.J. et al.

5 Nature 369:533-538 (1994); Lok, S. et al. Nature 369:565-568 (1994); Bartley, T.D. et al. Cell 77:1117-1124 (1994)). The encoded protein, termed thrombopoietin (TPO), stimulates proliferation and maturation of megakaryocytes and induces platelet production in vivo upon injection into
10 experimental animals.

Methods for the production and delivery of other proteins with therapeutic properties are desirable. For example, it has been demonstrated that recombinant β -interferon is an effective medication for treatment of
15 exacerbations in patients with relapsing-remitting multiple sclerosis (MS; see Kelley, C.L. and Smeltzer, S.C. J. Neuroscience Nursing 26:52-56 (1994)). Furthermore, it has been reported that β -interferon isolated from non-transfected cultured human fibroblasts may be an effective
20 means for preventing the progression of acute non-A, non-B hepatitis to chronic disease (Omata, M. et al., Lancet 338:914-915 (1991)).

As another example, it has been demonstrated that recombinant human DNase I is an effective agent for
25 reducing the viscosity of sputum from cystic fibrosis (CF) patients (Shak, S. et al., Proc. Natl. Acad. Sci. USA 87:9188-9192 (1990)) and for improving pulmonary function and decreasing exacerbations of respiratory disease in CF patients (Fuchs, H.J. et al., New Engl. J. Med. 331:637-642
30 (1994)). It has been further suggested that DNase I may be effective in improving respiratory function in patients with other respiratory diseases, such as chronic bronchitis and pneumonia (Shak, S. et al., op. cit.).

While TPO, β -interferon, and DNase I are useful, for
35 example, in the treatment of thrombocytopenia, MS, and CF,

-4-

respectively, production of therapeutic proteins using genetic engineering technology as taught in the prior art is limited to conventional recombinant DNA methods, in which the recombinant protein is purified from mammalian
5 cells expressing an exogenous cloned gene or cDNA under the control of a suitable promoter. The exogenous DNA encoding the protein of interest is introduced into cells in the form of a viral vector, circular plasmid DNA, or linear DNA fragment. Chinese Hamster Ovary (CHO) cell lines and their
10 derivatives (Gottesman, M. M. *Meth. Enzymol.* 151:3-8 (1987)) or mouse cell lines, such as NSO (Galfre, G. and Milstein, C., *Meth. Enzymol.* 73(B): 3-46 (1981)) or P3X63Ag8.653 (Kearney, J. et al. *J. Immunol.* 123: 1548-1550 (1979)) are commonly used, and the production of human therapeutic
15 proteins is thus accomplished by expression and purification of the protein from a cell of non-human origin.

In many cases, it is desirable to produce human therapeutic proteins in a human cell, for example, when it is desired that the glycosylation pattern of the protein be
20 similar to patterns normally found on human cells. In addition, the expression of human proteins in human cells is important in the development of gene therapy methods, in which a patient's cells are engineered to produce a desired therapeutic protein to alleviate the symptoms or cure a
25 disease.

Clearly, the development of novel methods for the production of these human proteins in human cells would be of benefit to patients, through the availability of a wider range of products with therapeutic effectiveness. One
30 approach proposed by scientists in the field for accomplishing this goal is to use homologous recombination, or gene targeting, to introduce a cloned, exogenous regulatory element (i.e. a promoter and/or enhancer) into a cell's genome at a pre-selected site such that the
35 regulatory element activates expression of a nearby gene,

-5-

ultimately resulting in production of the protein encoded by that gene. This approach has been suggested in U.S. Patent No. 5,272,071 and in foreign patent applications WO 91/06666, WO 91/06667 and WO 90/11354.

5 Summary of the Invention

Described herein are new methods for producing TPO, DNase I, and β -interferon through the generation of novel transcription units within a cell's genome, methods which differ dramatically from those in the art and represent a major advance in the ability to manipulate expression in mammalian cells. The methods are based on the fact that an exogenous regulatory sequence, an exogenous exon, either coding or non-coding, and a splice-donor site can be introduced into a preselected site in the genome by homologous recombination. The resulting cells are referred to as targeted or homologously recombinant cells. The introduced DNA is positioned such that transcripts under the control of the exogenous regulatory region include both the exogenous exon and endogenous exons present in either the TPO, DNase I, or β -interferon genes, resulting in transcripts in which the exogenous and endogenous exons are operatively linked. The novel transcription units produced by homologous recombination allow TPO, DNase I, or β -interferon to be produced in human cells using the naturally-occurring endogenous exons encoding these proteins without introducing any portion of the coding sequences of the cognate genes. The present invention further relates to improved materials and methods for both the in vitro production of TPO, β -interferon, and DNase I and for the production and delivery of TPO, β -interferon, and DNase I by gene therapy.

The methods of the present invention teach the production of TPO, β -interferon, or DNase I by gene activation, in which the coding DNA sequence of the

-6-

corresponding protein is not introduced into a cell by transfection of exogenous DNA encoding the protein. Instead, noncoding sequences upstream of one of these genes or coding or noncoding sequences within the genes are manipulated by gene targeting to create a novel transcription unit which expresses TPO, β -interferon, or DNase I. It is a purpose of this invention to define sequences upstream of the TPO, β -interferon, or DNase I genes, non-coding sequences (introns and 5' non-translated sequences) within the human TPO, β -interferon, or DNase I genes, and methods for utilizing these sequences for the production of TPO, β -interferon, or DNase I.

The methods described herein teach production of TPO, β -interferon, or DNase I proteins, by the generation of novel genes in which exogenous and endogenous exons are operatively linked. As a result of introduction of exogenous components into the chromosomal DNA of a cell, the expression of the protein encoded by the endogenous gene is activated. Other forms of altered gene expression may be envisioned, such as increasing expression of a gene which is expressed in the cell as obtained, changing the pattern of regulation or induction such that it is different than occurs in the cell as obtained, and reducing (including eliminating) expression of a gene which is expressed in the cell as obtained. For example, it may be desirable to perform in vitro protein production or gene therapy to produce a protein other than TPO, DNase I, or β -interferon using a cell type that naturally produces one of these proteins. In these settings, it would be desirable to eliminate expression of TPO, DNase I, or β -interferon.

The present invention further relates to DNA constructs useful in the method of activation of the TPO, β -interferon, or DNase I genes. The DNA constructs comprise: (a) targeting sequences; (b) a regulatory

-7-

sequence; (c) an exon; and (d) an unpaired splice-donor site. The targeting sequence in the DNA construct is derived from chromosomal DNA lying within and/or upstream of the desired gene and directs the integration of elements

5 (a) - (d) into the chromosomal DNA in a cell such that the elements (b) - (d) are operatively linked to sequences of the desired endogenous gene. In another embodiment, the DNA constructs comprise: (a) a targeting sequence, (b) a regulatory sequence, (c) an exon, (d) a splice-donor site,

10 (e) an intron, and (f) a splice-acceptor site, wherein the targeting sequence in the DNA construct is derived from chromosomal DNA lying within and/or upstream of the desired gene and directs the integration of elements (a) - (f) such that the elements of (b) - (f) are operatively linked to

15 the desired endogenous gene. The targeting sequence is homologous to the preselected site within or upstream of the *TPO*, β -interferon, or *DNase I* genes in the cellular chromosomal DNA with which homologous recombination is to occur. In the construct, the exon is generally 3' of the

20 regulatory sequence and the splice-donor site is 3' of the exon. Constructs of this type are disclosed in pending U.S. patent applications U.S.S.N. 07/985,586 and U.S.S.N. 08/243,391, all of which are incorporated herein by reference.

25 The following serves to illustrate two embodiments of the present invention, in which the sequences upstream of the *TPO* gene are altered to allow expression of *TPO* in primary, secondary, or immortalized cells which do not express *TPO* in detectable quantities in their untransfected

30 state as obtained. In embodiment 1 (Figure 1), the targeting construct contains two targeting sequences. Both the first and second targeting sequences are homologous to sequences upstream of the *TPO* coding region, with the first targeting sequence 5' of the second targeting sequence.

35 The targeting construct also contains a regulatory region,

-8-

an exon (which in this case, comprises noncoding sequences and begins at a CAP site) and an unpaired splice-donor site. The homologous recombination event that generates the novel transcription unit producing TPO is shown in

5 Figure 1.

In embodiment 2 (Figure 2), the targeting construct also contains two targeting sequences. The first targeting sequence is homologous to sequences upstream of the endogenous TPO coding region, and the second targeting
10 sequence is homologous to the second intron of the TPO gene. The targeting construct also contains a regulatory region, an exon (in this case a coding exon derived from the human growth hormone (hGH) gene) and an unpaired splice-donor site. The homologous recombination event that
15 generates the novel transcription unit producing TPO is shown in Figure 2.

In these two embodiments, the products of the targeting events are novel transcription units which generate a mature mRNA in which an exogenous exon is
20 positioned upstream of exon 2 (Embodiment 1) or exon 3 (Embodiment 2) of the endogenous TPO gene. The product of transcription, splicing, translation, and post-translational cleavage of the signal peptide is mature TPO. Embodiments 1 and 2 differ with respect to the relative
25 positions of the regulatory sequences of the targeting construct that are inserted and the specific pattern of splicing that needs to occur to produce the final, processed transcript.

The invention further relates to a method of
30 producing TPO, β -interferon, or DNase I in vitro or in vivo through introduction of a construct as described above into host cell chromosomal DNA by homologous recombination to produce a homologously recombinant cell. The homologously recombinant cell is then maintained under conditions which

-9-

will permit transcription, translation and secretion of TPO, β -interferon, or DNase I.

The present invention also relates to cells, such as homologously recombinant primary or secondary cells (i.e.,
5 non-immortalized cells) and homologously recombinant immortalized cells, useful for producing TPO, β -interferon, or DNase I, methods of making such cells, methods of using the cells for in vitro protein production, and methods of gene therapy. Homologously recombinant cells of the
10 present invention are of vertebrate origin, particularly of mammalian origin, and even more particularly of human origin. Homologously recombinant cells produced by the method of the present invention contain exogenous DNA which causes the homologously recombinant cells to express a
15 desired gene at a higher level or with a pattern of regulation or induction that is different than occurs in the corresponding cell that has not undergone homologous recombination.

In one embodiment, the activated TPO, β -interferon, or
20 DNase I gene can be further amplified by the inclusion of an amplifiable selectable marker gene which has the property that cells containing amplified copies of the selectable marker gene can be selected for by culturing the cells in the presence of the appropriate selectable agent.
25 The activated gene is amplified in tandem with the amplifiable selectable marker gene. Cells containing many copies of the activated gene are useful for in vitro protein production and gene therapy.

Homologously recombinant cells of the present
30 invention are useful in a number of applications in humans and animals. In one embodiment, the cells can be implanted into a human or an animal for protein delivery in the human or animal. For example, TPO, DNase I, or β -interferon can be delivered systemically or locally in humans for
35 therapeutic benefit in the treatment of disease (TPO for

-10-

thrombocytopenia, DNase I for CF, or β -interferon for the treatment of MS). In addition, homologously recombinant non-human cells producing TPO, DNase I, or β -interferon of non-human origin may be produced, and human or non-human
5 cells expressing TPO, DNase I, or β -interferon may be enclosed within barrier devices and implanted into humans or animals for use in a therapy.

Brief Description of the Drawings

Figure 1 is a schematic diagram of a strategy for
10 transcriptionally activating the TPO gene by the creation of a novel transcription unit; thick lines: targeting sequences; thin lines: introns and 5' upstream region; cross-hatched box, regulatory sequence; stippled boxes: noncoding exon sequences; black boxes: coding exon
15 sequences; open boxes: splice sites. The splice-donor site (SD) of the exogenous exon in the targeting construct and the splice-acceptor site (SA) flanking TPO exon 2 which is involved in splicing to the exogenous exon are indicated.

Figure 2 is a schematic diagram of a strategy for
20 transcriptionally activating the TPO gene by the creation of a novel transcription unit; thick lines: targeting sequences; thin lines: intron 1 and 5' upstream region; cross-hatched box: regulatory sequence; stippled boxes: noncoding exon sequences; black boxes: coding exon
25 sequences; open boxes, splice sites. The splice-donor site (SD) of the exogenous exon in the targeting construct and the splice-acceptor site (SA) flanking TPO exon 3 which is involved in splicing to the exogenous exon are indicated.

Figure 3 presents the 6,943 bp genomic XbaI fragment
30 encompassing the 5' flanking region and exons 1, 2, and 3 of the human thrombopoietin (TPO) gene. The XbaI fragment is depicted by the solid line, while exons 1, 2, and 3 are represented by the solid boxes. The nucleotide positions of the ApaI, BamHI, HindIII, EcoRI, NotI, SfiI and XbaI

-11-

recognition sequences are indicated. Nucleotides are numbered starting at the hTPO ATG initiation codon.

Figures 4A-4D present the nucleotide sequence of 4,488 bp of genomic DNA (SEQ ID NO: 3) from the human TPO locus lying 5' to the known cDNA sequence (de Sauvage et al., op. cit.). Nucleotide numbers are noted at the beginning of each line. Numbering is based on the ATG initiation codon at position 1 (see Figures 5A-5B). Ambiguities in the nucleotide sequence are represented using the following code: R = A or G (purine); H = A, C, or T; V = A, C, or G; N_ = A, C, G, or T; K = G or T; S = G or C; W = A or T. The recognition sites for *ApaI*, *BamHI*, *HindIII*, *NotI*, *SfiI* and *XbaI* and their corresponding nucleotide positions are indicated above the sequence.

Figures 5A-5B present the nucleotide sequence of 2,455_bp of genomic DNA (SEQ ID NO: 4) from the human TPO locus extending downstream from the position of the 5' end of the known cDNA sequence (de Sauvage et al., op. cit.). Nucleotide numbers are noted at the beginning of each line. Numbering is based on the ATG initiation codon at position 1. Shown are exon 1, intron 1, exon 2, intron 2, exon 3, and a portion of intron 3. Exons 1, 2, and 3 are underlined, and the coding portions of exons 2 and 3 are noted as underlined triplets. The intron-exon boundaries are deduced from the published cDNA sequence (de Sauvage et al., op. cit.). The recognition sites for *ApaI*, *EcoRI*, and *XbaI* and their corresponding nucleotide positions are indicated above the sequence.

Figure 6 is a schematic diagram of the strategy for activating the human TPO gene using targeting construct pTPO1 as described in Example 2. The positions of the *dhfr* and *neo* markers, the exogenous CMV promoter and TPO exons 1-3 are indicated. Thick lines: targeting sequences; thin lines: introns and 5' upstream region; cross-hatched box: CMV promoter; stippled boxes: noncoding exon sequences;

-12-

black boxes: coding exon sequences; open boxes, splice sites. The splice-donor site (SD) of the exogenous exon in the targeting construct and the splice-acceptor site (SA) flanking TPO exon 3 which is involved in splicing to the exogenous exon are indicated. Recognition sites for BamHI (B), NotI (N), ClaI (C), XhoI (X), and XbaI which are relevant to the construction of the targeting construct are marked.

Figure 7 is a schematic diagram of the strategy for activating the human TPO gene using targeting construct pTPO2 as described in Example 2. The positions of the dhfr and neo markers, the exogenous CMV promoter and TPO exons 1-3 are indicated. Thick lines: targeting sequences; thin lines: introns and 5' upstream region; cross-hatched box: CMV promoter; heavily stippled boxes: noncoding exons from the CMV IE gene; lightly stippled boxes: noncoding exon sequences of TPO exons 1 and 2; black boxes: coding exon sequences of TPO exons 2 and 3; open boxes: splice sites. The splice-donor (SD) and splice-acceptor (SA) sites flanking the noncoding exons in the targeting construct and the splice-acceptor site (SA) flanking TPO exon 2 which is involved in splicing to the unpaired splice-donor site of the 3' exogenous exon are indicated. Recognition sites for BamHI (B), HindIII (H), NotI (N), ClaI (C), SalI (S), EcoRI (R), and XbaI which are relevant to the construction of the targeting construct are marked.

Figure 8 is a schematic diagram of the strategy for activating the human TPO gene using targeting construct pTPO3 as described in Example 2. The positions of the dhfr and neo markers, the exogenous CMV promoter and TPO exons 1-3 are indicated. Thick lines: targeting sequences; thin lines: introns and 5' upstream region; cross-hatched box: CMV promoter; stippled boxes: noncoding exon sequences of TPO exons 1 and 2; black boxes: coding exon sequences (the coding exon corresponding to hGH exon 1 in the targeting

-13-

construct and in the novel transcription unit is marked); open boxes: splice sites. The splice-donor site (SD) of the exogenous exon in the targeting construct and the splice-acceptor site (SA) flanking TPO exon 3 which is involved in splicing to the exogenous exon are indicated. Recognition sites for *Bam*HI (B), *Hind*III (H), *Cla*I (C), *Xho*I (X), *Eco*RI (R), and *Xba*I which are relevant to the construction of the targeting construct are marked.

Figure 9 is a diagrammatic representation of the approximately 8 kb *Hinc*II fragment encompassing the 5' flanking region, exons 1 and 2, and the sequences downstream of exon 2 of the human *DNase I* gene. The *Hinc*II fragment is depicted by the solid line, while exons 1 and 2 are represented by solid rectangular boxes. The nucleotide positions of the *Apa*I, *Bam*HI, *Hinc*II, *Esp*I, *Sph*I and *Sma*I recognition sequences are indicated. Nucleotides are numbered starting at the AUG initiation codon. The nucleotide positions which reside upstream of exon 2 are based on the DNA sequence presented in Figures 10 and 11.

Figures 10A-10D present the nucleotide sequence encompassing 4,042 bp of DNA (SEQ ID NO: 17) from the human *DNase I* locus lying 5' to the known cDNA sequence (Shak, S. et al. op. cit.). Nucleotides numbers are noted at the beginning of each line. Numbering is based on the ATG initiation codon at position 1 (see Figure 11). The recognition sites, and the corresponding nucleotide positions for *Apa*I, *Bam*HI, *Hinc*II, *Esp*I, and *Sph*I are indicated above the sequence.

Figure 11 presents the nucleotide sequence of 810 bp of DNA (SEQ ID NO: 18) from the human *DNase I* locus extending downstream from the position of the 5' end of the known cDNA sequence (Shak, S. et al. op. cit.). Shown are exon 1, intron 1, and a portion of exon 2. Exon 1 and 2 sequences are underlined and the coding sequences are noted as underlined triplets. The positions of the putative CAP

-14-

site and the AUG initiation codon are indicated. The intron-exon boundaries are deduced from the published cDNA sequence (Shak S. et al., op. cit.).

Figure 12 shows a strategy for activation of the human DNase I gene by homologous recombination. The targeting fragment is a 4633 bp *Bam*HI fragment from pDNaseI which contains; 283 bp of 5' targeting sequence from position -1162 (*Bam*HI site) to -860 (*Apa*I site), an amplifiable *dhfr* expression unit, *neo* gene, CMV IE promoter, a CAP site, a non-codon exon, an unpaired splice-donor site and 363 bp of 3' targeting sequence from position -860 (*Esp*I site) to -468 (*Bam*HI site). The *dhfr* expression unit and the *neo* gene are depicted by open arrows, the orientation of the arrows represent the direction of transcription. The positions of the CMV promoter, TATA box, CAP site and splice donor sequence (SD) are indicated. Activation of the DNase I gene is achieved by integration of the targeting fragment into the genome of the recipient cells by homologous recombination. The targeted gene product is depicted in the lower panel of the figure. The mRNA precursor which includes a non-coding 5' exon, a chimeric intron and exon 2 of the DNase gene, is represented by the thin arrow.

Figure 13 is a diagrammatic representation of 9,939 bp encompassing the 5' flanking region, coding sequence and the 3' untranslated region of the human β -interferon gene. The 5' and 3' flanking regions are depicted by the solid line and the transcribed region is represented by the solid box. The nucleotide positions of the *Bal*I, *Bgl*II, *Eco*RI and *Pvu*II recognition sequences are indicated. Nucleotides are numbered starting at the β -interferon ATG translational initiation codon (see Figure 15).

Figures 14A-14G present the nucleotide sequence of 8,355 bp of DNA (SEQ ID NO: 23) from the human β -interferon locus lying 5' to the known sequence (GenBank HUMIFNB1F).

-15-

Nucleotide numbers are noted at the beginning of each line. Numbering is based on the ATG initiation codon at position 1 (see Figures 15). The recognition sites for *Bgl*II, *Eco*RI and *Pvu*II and their corresponding nucleotide positions are indicated above the sequence.

Figures 15A-15B present the nucleotide sequence of 1,584 bp of DNA (SEQ ID NO: 24) from the human β -interferon locus extending downstream from the 5' end of the known sequence (GenBank HUMIFNB1F). Nucleotide numbers are noted at the beginning of each line. Numbering is based on the ATG initiation codon at position 1. The transcribed region is underlined and the coding sequences are noted as underlined triplets. The position of the CAP site and AUG initiation codon are indicated. The recognition sites for *Bal*I, *Bgl*II and *Pvu*II and their corresponding nucleotide positions are indicated above the sequence.

Figure 16 depicts the strategy for activation of the human β -interferon gene by homologous recombination using targeting construct pIFNb-1 as described in Example 7. The positions of the TATA box, CAP site, *dhfr* and *neo* markers, the exogenous CMV promoter, and the β -interferon 5' flanking region and coding sequence are indicated. Thick lines: targeting sequences; thin lines: intron, β -interferon 5' and 3' non-coding sequences; solid box: CMV promoter; shaded box: endogenous β -interferon transcribed region; cross-hatched box: non-coding CMV exon 1 and the chimeric exon 2. The splice-donor site (SD) of the exogenous exon and the splice-acceptor site (SA) flanking the chimeric exon 2 are indicated. Recognition sites for *Bam*HI, *Eco*RI, *Hinc*II, *Nde*I and *Pvu*II which are relevant to the construction of the targeting construct are marked.

Detailed Description of the Invention

The present invention as set forth above, relates to a method of expressing TPO, DNase I, or β -interferon in human

-16-

cells by activation of the endogenous TPO, DNase I, or β -interferon genes. In the present invention, homologous recombination is used to insert a regulatory region, an exon, and a splice-donor site upstream of endogenous exons coding for TPO, DNase I, or β -interferon, generating novel transcription units which are active in the homologously recombinant cell produced. The present invention further relates to homologously recombinant cells produced by the present method and to uses of the homologously recombinant cells. In a related embodiment, an activated TPO, DNase I, or β -interferon gene is amplified subsequent to activation, thus allowing enhanced expression of the activated gene.

The invention is based upon the discovery that the regulation or activity of endogenous genes of interest in a cell can be altered by creating a novel gene, in which the transcription product of the gene combines exogenous and endogenous exons and is under the control of an exogenous promoter. The method is practiced by inserting into a cell's genome, at a preselected site, through homologous recombination, DNA constructs comprising: (a) one or more targeting sequences; (b) a regulatory sequence; (c) an exon and (d) an unpaired splice-donor site, wherein the targeting sequence or sequences are derived from chromosomal DNA within and/or upstream of a desired endogenous gene and directs the integration of elements (a) - (d) such that the elements (b) - (d) are operatively linked to the endogenous gene. In another embodiment, the DNA constructs comprise: (a) one or more targeting sequences, (b) a regulatory sequence, (c) an exon, (d) a splice-donor site, (e) an intron, and (f) a splice-acceptor site, wherein the targeting sequence or sequences are derived from chromosomal DNA within and/or upstream of a desired endogenous gene and directs the integration of elements (a) - (f) such that the elements of (b) - (f) are operatively linked to the first exon of the endogenous gene.

-17-

The present invention relates particularly to novel DNA sequences that can be used in the construction of targeting constructs. Non-coding genomic DNA sequences within and upstream of the transcribed regions of the *TPO* and *DNase I* genes, and upstream of the transcribed region of the β -interferon gene, were cloned and are described for the first time. These sequences or DNA fragments comprising these sequences may be used as targeting sequences in DNA constructs useful for gene activation by homologous recombination. Typically, a targeting sequence is at least about 20 base pairs in length. The size of the sequence is chosen to be a size which selectively promotes homologous recombination with desired genomic DNA sequences.

Analysis of the genomic DNA sequences and comparison to the known cDNA sequences revealed features essential for the construction of targeting constructs. For example, for the first time, it is shown that the first exon of the human *TPO* gene is entirely non-coding, and that translation initiates within the second exon of the endogenous gene. This information was important to the design of the gene activation constructs described herein, in which splicing of an exogenous exon to the endogenous second exon requires that the exogenous exon be non-coding, or in which splicing of an exogenous coding exon requires that targeting be performed such that the exogenous coding exon is inserted in a position so that it can be spliced to the endogenous third exon of the *TPO* gene. Furthermore, the cloning of approximately 6.3 kb of DNA sequence from upstream of the human *TPO* gene provided targeting sequences useful for the development of gene activation constructs. Figure 4 shows approximately 4.5 kb of novel DNA sequence from the human *TPO* locus lying 5' of the known cDNA sequence (de Sauvage, F. J. et al., op. cit.). Figure 5 shows approximately 2.5 kb of DNA sequence from the human *TPO* locus extending in the 3' direction from the 5' boundary of the known cDNA

-18-

sequence. Intron sequences (positions -1815 to -145, positions 14 to 245, and positions 374 to 570) of Figure 5 are novel. DNA constructs comprising the novel sequences of Figures 4 and 5, or fragments derived from these sequences, are useful for homologous recombination as taught herein.

Similarly, for the first time it is shown that the first exon of the human *DNase I* gene is entirely non-coding. This information was important to the design of the targeting constructs described herein. Example 5, for example, describes a targeting construct which includes two non-coding exons separated by an intron, and which is inserted upstream of *DNase I* exon 1. This configuration allows promoter position to be optimized by varying the length of either the exogenous intron or the intron present between the exogenous exon and the endogenous second exon of the *DNase I* gene, while ensuring that the primary transcript will be spliced appropriately and that translation initiates at the correct position for synthesis of functional *DNase I*. Furthermore, the cloning of approximately 4.5 kb of DNA sequence from upstream of the human *DNase I* gene provided targeting sequences useful for the development of gene activation constructs. Figure 10 shows approximately 4 kb of novel DNA sequence from the human *DNase I* locus lying 5' of the known cDNA sequence (Shak, S. et al. op. cit.). Figure 11 shows approximately 0.8 kb of DNA sequence from the human *DNase I* locus extending in the 3' direction from the 5' boundary of the known cDNA sequence. Intron sequences (positions -328 to -2) of Figure 11 are novel. DNA constructs comprising the novel sequences of Figures 10 and 11, or fragments derived from these sequences, are useful for homologous recombination as described herein.

Finally, the analysis of the upstream region of the β -interferon gene (a gene which is known to lack introns)

-19-

was cloned and sequenced and a detailed restriction map was produced. Previously, only 357 bp of DNA upstream of the translation initiation codon was characterized (see Genbank entry HUMIFNB1F). The cloning and sequence analysis
5 provided approximately 9.6 kb of genomic DNA upstream of the gene for the design and construction of a targeting construct (Example 7). Figure 14 shows approximately 8.4 kb of novel DNA sequence from the β -interferon locus lying 5' of the known sequences (Genbank entry HUMIFNB1F).
10 DNA constructs comprising the novel sequences of Figure 14, or fragments derived from these sequences, are useful for homologous recombination as taught herein.

The following defines the DNA constructs of the present invention, the elements comprising the DNA
15 constructs of the present invention (Section A), methods in which the DNA constructs are used to produce homologously recombinant cells (Section B), the structure of the targeted gene and the resulting product (Section C), the homologously recombinant cells produced (Section D), uses
20 of these cells (Sections E and F), and the advantages of the constructs and methods described herein (Section G).

A. The DNA Construct

The DNA constructs of the present invention include at least the following components: a targeting sequence; a
25 regulatory sequence; an exon and a splice-donor site. In the construct, the exon is 3' of the regulatory sequence and the splice-donor site is 3' of the exon. In addition, there can be multiple exons and/or introns preceding (5' to) the exon flanked by the splice-donor site. Taken as a
30 group, the exons, introns, and splice-sites are referred to as the "structural elements" of the construct, so-called because they are important in defining the structure of the novel gene produced by homologous recombination between genomic DNA and DNA of the targeting construct. As

-20-

described herein, there frequently are additional construct components, such as a selectable and/or amplifiable markers.

The DNA in the construct is referred to as exogenous DNA, defined herein as DNA which is introduced into a cell by the methods described herein, such as with the DNA constructs of the present invention. Exogenous DNA can contain sequences identical to or different from the endogenous DNA. The term endogenous DNA is defined herein as DNA present in the cell as obtained.

The DNA of the construct can be obtained from sources in which it occurs in nature or can be produced, using genetic engineering techniques or synthetic processes.

1. The Targeting Sequence

The targeting sequence or sequences are DNA sequences which permit homologous recombination into the genome of the selected cell containing the gene of interest. Targeting sequences are, generally, DNA sequences which are homologous to (i.e., identical or sufficiently similar to) DNA sequences present in the genome of the cells as obtained (e.g., coding or noncoding DNA, located upstream of the transcriptional start site, within the transcribed region encompassing the gene, or downstream of the transcriptional stop site of the gene, or sequences present in the genome through a previous modification), such that the targeting sequence and cellular DNA can undergo homologous recombination. In general, two sequences are described as homologous if a DNA strand of one sequence is capable of hybridizing to a DNA strand of the other sequence under conditions standardly used for the detection of sequence similarity (see, for example, Ausubel et al., *Current Protocols in Molecular Biology*, Wiley, New York, NY. (1987)). The targeting sequence or sequences used are selected with reference to the site into which the DNA in

-21-

the DNA construct is to be inserted and may be derived from either genomic or cDNA sequences. Typically, a targeting sequence is at least about 20 base pairs in length. The size of the sequence is chosen to be a size which

5 selectively promotes homologous recombination with desired genomic DNA sequences.

One or more targeting sequences can be employed. For example, a circular plasmid or DNA fragment preferably employs a single targeting sequence. A linear plasmid or

10 DNA fragment preferably employs two targeting sequences with exogenous DNA to be inserted into genome positioned between the two targeting sequences. The targeting sequence or sequences can be within an endogenous gene (e.g., within the sequences of an exon and/or intron),

15 within the endogenous promoter sequences, or upstream of the endogenous promoter sequences. The targeting sequence or sequences can include those regions of a gene presently known or sequenced and/or regions further upstream which are structurally uncharacterized but can be mapped using

20 restriction enzymes and cloning approaches available to one skilled in the art.

2. The Regulatory Sequence

The regulatory sequence of the DNA construct can be comprised of one or more of a variety of elements,

25 including: promoters (such as a constitutive or inducible promoters), enhancers, scaffold-attachment regions or matrix attachment regions, (McKnight, R.A. et al., *Proc. Natl. Acad. Sci. USA* 89:6943-6947 (1992); Phi-Van, L. and Strätling, W.H. *EMBO J.* 7:655-664 (1988)) negative

30 regulatory elements, locus control region, (Pondel, M.D. et al., *Nucl. Acids Res.* 20:237-243 (1992); Li, Q. and Stamatoyannopoulos, G. *Blood* 84:1399-1401 (1994)) transcription factor binding sites, or combinations of said sequences.

-22-

3. Structural Elements of the DNA Construct

a. Exons and Introns

An exon is defined herein as a DNA sequence which is copied into RNA and is present in a mature mRNA molecule.

- 5 An intron is defined as a sequence of one or more nucleotides lying between two exons and which is removed, by splicing, from a precursor RNA molecule in the formation of an mRNA molecule.

The DNA constructs of the present invention contain
10 one or more exons. The exons can, optionally, contain DNA which encodes one or more amino acids and/or partially encodes an amino acid (i.e., one or two bases of a codon). Where the exogenous exon or exons encode one or more amino acids and/or a portion of an amino acid, the DNA construct
15 is designed such that, upon transcription and splicing, the reading frame is in-frame with the second or subsequent exon of the endogenous gene's coding region. As used herein, in-frame means that the encoding sequences of, for example, a first exon and a second exon when fused, join
20 together nucleotides in a manner that does not change the appropriate reading frame of the portion of the mRNA derived from the second exon.

In the case of activating the *TPO* and *DNase I* genes, the exogenous exon can, preferably, be derived from any
25 gene in which the exon includes a CAP site and non-coding sequences. Examples would include the first exon of the CMV immediate-early gene and follicle stimulating hormone (FSH) gene. In the case of β -interferon, whose gene contains no natural introns, there are preferably two
30 exogenous non-coding exons, separated by an intron, in the targeting construct.

b. Splice-Sites

Introns contained within the mRNA of eukaryotic cells are removed through the recognition of signals termed

-23-

splice-donor and splice-acceptor sites. A splice-donor site is a sequence which directs the splicing of one exon to another exon. Typically, the first exon lies 5' of the second exon, and the splice-donor site overlapping and
5 flanking the first exon on its 3' side recognizes a splice-acceptor site flanking the second exon on the 5' side of the second exon. Splice-donor sites have a characteristic consensus sequence represented as:
(A/C)AGGURAGU (where R denotes a purine nucleotide) with
10 the GU in the fourth and fifth positions being required (Jackson, I.J., *Nucleic Acids Research* 19: 3715-3798 (1991)). The first three bases of the splice-donor consensus site are the last three bases of the exon. Splice-donor sites are functionally defined by their
15 ability to effect the appropriate reaction within the mRNA splicing pathway.

An unpaired splice-donor site is defined herein as a splice-donor site which is present in a targeting construct and is not accompanied in the targeting construct by a
20 splice-acceptor site positioned 3' to the unpaired splice-donor site. Upon homologous recombination between the targeting sequences and genomic DNA, the unpaired splice-donor site results in splicing to an endogenous splice-acceptor site.

25 A splice-acceptor site is a sequence which, like a splice-donor site, directs the splicing of one exon to another exon. Acting in conjunction with a splice-donor site, the splicing apparatus uses a splice-acceptor site to effect the removal of an intron. Splice-acceptor sites
30 have a characteristic sequence represented as:
YYYYYYYYNYAG, where Y denotes any pyrimidine and N denotes any nucleotide (Jackson, I.J., *Nucleic Acids Research* 19:3715-3798 (1991)).

-24-

c. Marker Genes for Selection and Amplification

The identification of the targeting event can be facilitated by the use of one or more selectable marker genes typically contained within the targeting DNA construct. The use of both positively and negatively selectable markers for identifying targeted events is described in related pending applications U.S.S.N. 08/243,391, U.S.S.N. 07/985,586, U.S.S.N. 07/789,188, PCT/US93/11704, and PCT/US92/09627.

10 Homologously recombinant cells containing multiple copies of the novel transcription units produced by the present invention may be isolated by including within the targeting DNA construct an amplifiable marker gene which has the property that cells containing multiple copies of

15 the selectable marker gene can be selected for by culturing the cells in the presence of an appropriate selectable agent. The novel transcription unit will be amplified in tandem with the amplified selectable marker gene, allowing the production of very high levels of the desired protein.

20 Amplifiable marker genes and their use are described in applications U.S.S.N. 08/243,391, U.S.S.N. 07/985,586, and PCT/US93/11704.

In one embodiment the positively selectable marker *neo* is used (derived from the bacterial *neomycin*

25 *phosphotransferase* gene) is used to select for cells which have stably incorporated the DNA of the targeting construct, and the mouse *dhfr* (*dihydrofolate reductase*) gene is used to subsequently amplify the novel transcription unit present in homologously recombinant

30 cells.

d. Additional Elements of the Targeting Construct

As taught herein, gene targeting can be used to insert a regulatory sequence within an endogenous gene (e.g.,

-25-

within the sequences of an exon and/or intron), within the endogenous promoter sequences, or upstream of the endogenous promoter sequences, with said genes corresponding to the endogenous cellular *TPO*, β -interferon, or *DNase I* gene. Alternatively or additionally, the targeting constructs may be designed to include sequences which affect the structure or stability of the *TPO*, β -interferon, or *DNase I* protein or corresponding RNA molecule. For example, RNA stability elements, splice sites, and/or leader sequences of RNA molecules can be modified to improve or alter the function, stability, and/or translatability of an RNA molecule. Protein sequences may also be altered, such as signal sequences, active sites, and/or structural sequences for enhancing or modifying glycosylation, transport, secretion, or functional properties of a protein. According to this method, introduction of the exogenous DNA results in the alteration of the structural or functional properties of the expressed proteins or RNA molecules.

In one embodiment the method can be used to create novel transcription units encoding fusion proteins in which structural, enzymatic, or ligand or receptor binding protein domains of another protein are fused to *TPO*, *DNase I*, or β -interferon. In these cases the exogenous coding DNA contains an ATG translation initiation codon in-frame with the coding sequences of the endogenous *TPO*, *DNase I*, or β -interferon gene. For example, the exogenous DNA can encode a sequence which can anchor *TPO* or *DNase I* to a membrane, a portion of a signal peptide designed to improve cellular secretion, leader sequences, enzymatic regions, transmembrane domain regions, co-factor binding regions, or other functional regions.

The DNA construct can also include a bacterial origin of replication and bacterial antibiotic resistance markers or other selectable markers, which allow for large-scale

plasmid propagation in bacteria or any other suitable cloning/host system.

B. Transfection and Homologous Recombination

According to the present method, the construct is introduced into the cell, such as a primary, secondary, or immortalized cell, as a single DNA construct, or as separate DNA sequences which become incorporated into the chromosomal or nuclear DNA of a transfected cell.

The targeting DNA construct can be introduced into cells on a single DNA construct or on separate constructs. The total length of the DNA construct will vary according to the number of components and the length of each and the construct will generally be at least about 200 nucleotides. Further, the DNA can be introduced as linear, double-stranded (with or without single-stranded regions at one or both ends), single-stranded, or circular DNA.

Any of the construct types of the disclosed invention is then introduced into the cell to obtain a transfected cell. The transfected cell is maintained under conditions which permit homologous recombination, as is known in the art (reviewed in Capecchi, M.R., *Science* 244:1288-1292 (1989)). When the homologously recombinant cell is maintained under conditions sufficient for transcription of the DNA, the regulatory region introduced by the targeting construct, as in the case of a promoter, will activate expression of the novel transcription unit produced by homologous recombination.

The DNA constructs may be introduced into cells by a variety of physical or chemical methods, including electroporation, microinjection, microprojectile bombardment, calcium phosphate precipitation, and liposome-, polybrene-, or DEAE dextran-mediated transfection.

-27-

C. The Targeted Gene and Resulting Product

The targeting DNA construct, when introduced by homologous recombination or targeting into cells containing the *TPO*, β -interferon, or *DNase I* gene, produces a novel
5 transcription unit which results in the expression of *TPO*, β -interferon, or *DNase I*.

At the targeted site in the genome, the exogenous regulatory sequence is operatively linked to a CAP site, which initiates transcription. Operatively linked is
10 defined as a configuration in which the exogenous regulatory sequence, exon, splice-donor site and, optionally, an intron sequence and splice-acceptor site, are appropriately targeted at a position relative to the endogenous gene such that the regulatory element directs
15 the production of a primary RNA transcript which initiates at a CAP site and includes sequences corresponding to the exogenous exon or exons and endogenous exons the *TPO*, *DNase I*, or β -interferon gene. In an operatively linked configuration the splice-donor site of the targeting
20 construct directs a splicing event between an exogenous exon and the splice-acceptor site of an endogenous exon, such that a desired protein can be produced from the fully spliced mature transcript. In one embodiment, the splice-acceptor site is endogenous, such that the splicing
25 event is directed to an endogenous exon of the *TPO* or *DNase I* gene. In another embodiment an intron and a splice-acceptor site are included in the targeting construct used to activate the β -interferon gene, and a splicing event removes the intron introduced by the targeting construct.

30 D. The Homologously Recombinant Cells

The targeting event results in the insertion of the regulatory and structural sequences of the targeting construct into a cell's genome, creating a novel

-28-

transcriptional unit under the control of the exogenous regulatory sequences.

Homologous recombination between the genomic DNA and the introduced DNA results in a homologously recombinant cell, which may be a primary, secondary, or immortalized human or other mammalian cell in which sequences which alter the expression of an endogenous gene are operatively linked to the endogenous TPO, DNase I, or β -interferon gene. Particularly, the invention includes a homologously recombinant cell comprising exogenous regulatory sequences and an exon, flanked by a splice-donor site, which are introduced at a predetermined site by a targeting DNA construct, and are operatively linked to the coding region of the endogenous gene. Optionally, there may be multiple exogenous exons (coding or non-coding) and introns operatively linked to any exon of the endogenous gene. The resulting homologously recombinant cells are cultured under conditions which select for amplification, if appropriate, of the DNA encoding the amplifiable marker and the novel transcriptional unit. With or without amplification, cells produced by this method can be cultured under conditions, as are known in the art, suitable for the expression of TPO, β -interferon, or DNase I.

The targeting constructs and methods of the present invention may be used with, for example, primary or secondary cell strains (which exhibit a finite number of mean population doublings in culture and are not immortalized) and immortalized cell lines (which exhibit an apparently unlimited lifespan in culture). Primary and secondary cells include, for example, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Where the

-29-

homologously recombinant cells are to be used in gene therapy, primary cells are preferably obtained from the individual to whom the resulting homologously recombinant cells are administered. However, primary cells can be
5 obtained from a donor (other than the recipient) of the same species. Examples of immortalized human cell lines which may be used with the DNA constructs and methods of the present invention include, but are not limited to, HT1080 cells (ATCC CCL 121), HeLa cells and derivatives of
10 HeLa cells (ATCC CCL 2, 2.1 and 2.2), MCF-7 breast cancer cells (ATCC BTH 22), K-562 leukemia cells (ATCC CCL 243), KB carcinoma cells (ATCC CCL 17), 2780AD ovarian carcinoma cells (Van der Blick, A.M. et al., *Cancer Res*, 48:5927-5932 (1988), Raji cells (ATCC CCL 86), WiDr colon adenocarcinoma
15 cells (ATCC CCL 218), SW620 colon adenocarcinoma cells (ATCC CCL 227), Jurkat cells (ATCC TIB 152), Namalwa cells (ATCC CRL 1432), HL-60 cells (ATCC CCL 240), Daudi cells (ATCC CCL 213), RPMI 8226 cells (ATCC CCL 155), U-937 cells (ATCC CRL 1593), Bowes Melanoma cells (ATCC CRL 9607),
20 WI-38VA13 subline 2R4 cells (ATCC CLL 75.1), and MOLT-4 cells (ATCC CRL 1582), as well as heterohybridoma cells produced by fusion of human cells and cells of another species. Secondary human fibroblast strains, such as WI-38 (ATCC CCL 75) and MRC-5 (ATCC CCL 171) may be used.
25 Further discussion of the types of cells that may be used in practicing the methods of the present invention is presented in applications U.S.S.N. 08/243,391, U.S.S.N. 07/985,586, U.S.S.N. 07/789,188, U.S.S.N. 07/911,533, U.S.S.N. 07/787,840, PCT/US93/11704, and PCT/US92/09627.

30 E. In Vivo Protein Production

Homologously recombinant cells of the present invention in which the expression properties of the endogenous *TPO*, β -interferon, or *DNase I* gene are altered are useful in gene therapy, as populations of homologously

-30-

recombinant cell lines, as populations of homologously recombinant primary or secondary cells, homologously recombinant clonal cell strains or lines, homologously recombinant heterogenous cell strains or lines, and as cell mixtures in which at least one representative cell of one of the preceding categories of homologously recombinant cells is present. Homologously recombinant primary cells, clonal cell strains or heterogenous cell strains are administered to an individual in whom the abnormal or undesirable condition is to be treated or prevented, in sufficient quantity and by an appropriate route, to express or make available the desired product at physiologically relevant levels. A physiologically relevant level is one which either approximates the level at which the product is normally produced in the body or results in improvement of the abnormal or undesirable condition. Methods for gene therapy in which homologously recombinant cells are introduced into an individual for the purpose of in vivo protein production are described in pending applications U.S.S.N. 08/243,391, U.S.S.N. 07/985,586, U.S.S.N. 07/789,188, U.S.S.N. 07/911,533, U.S.S.N., PCT/US93/11704, and PCT/US92/09627.

In one embodiment, the invention relates to a method of providing TPO to a mammal introducing homologously recombinant cells into the mammal in sufficient number to produce an effective amount of TPO in the mammal.

In another embodiment homologously recombinant cells expressing DNase I can be administered to the trachea and lungs of a cystic fibrosis patient, for the purpose of in vivo secretion of DNase I for the relief of respiratory distress.

In a third embodiment, homologously recombinant cells expressing β -interferon may be implanted into a patient suffering from multiple sclerosis, for the purpose of in

-31-

vivo secretion of β -interferon to diminish exacerbations associated with the disease.

F. In Vitro Protein Production

Homologously recombinant cells produced according to this invention can also be used for in vitro production of TPO, β -interferon, or DNase I. The cells are maintained under conditions, as are known in the art, which result in expression of the protein. Proteins expressed using the methods described may be purified from cell lysates or cell supernatants. Proteins made according to this method can be prepared as a pharmaceutically-useful formulation and delivered to a human or non-human animal by conventional pharmaceutical routes as is known in the art (e.g., oral, intravenous, intramuscular, intranasal, intratracheal or subcutaneous). As described herein, the homologously recombinant cells can be immortalized, primary, or secondary human cells. The use of cells from other species may be desirable in cases where the non-human cells are advantageous for protein production purposes where the non-human TPO, DNase I, or β -interferon produced is useful therapeutically.

G. Advantages

The methodologies, DNA constructs, cells, and resulting proteins of the invention herein possess versatility and many other advantages over processes currently employed within the art in gene targeting. The ability to activate expression of an endogenous TPO, β -interferon, or DNase I gene by positioning an exogenous regulatory sequence and other structural sequences at various positions ranging from directly fused to portions of the normal gene's coding region to 30 kilobase pairs or further upstream of the transcribed region of an endogenous gene, or within an intron of an endogenous gene, is

-32-

advantageous for gene expression in cells. For example, it can be employed to position the regulatory element upstream or downstream of regions that normally silence or negatively regulate a gene. The positioning of a regulatory element upstream or downstream of such a region can override such dominant negative effects that normally inhibit transcription. In addition, regions of DNA that normally inhibit transcription or have an otherwise detrimental effect on the expression of a gene may be deleted using the targeting constructs, described herein. The present invention also allows proteins to be expressed in the context of their normal intron sequences, which have been shown to be important factors in the expression of genes in mammalian cells (cf. Korb. M. et al. Nucl. Acids Res. 21: 5901-5908 (1993)).

Additionally, since promoter function is known to depend strongly on the local environment, a wide range of positions may be explored in order to find those local environments optimal for function. However, since, ATG start codons are found frequently within mammalian DNA (approximately one occurrence per 48 base pairs as calculated from nearest-neighbor dinucleotide frequencies in human DNA), transcription cannot simply initiate at any position upstream of a gene and produce a transcript containing a long leader sequence preceding the correct ATG start codon, since the frequent occurrence of ATG codons in such a leader sequence will prevent translation of the correct gene product and render the message useless. Thus, the incorporation of an exogenous exon, a splice-donor site, and, optionally, an intron and a splice-acceptor site into targeting constructs comprising a regulatory region allows gene expression to be optimized by identifying the optimal site for regulatory region function, without the limitation imposed by needing to avoid inappropriate ATG start codons in the mRNA produced. This provides

-33-

significantly increased flexibility in the placement of the construct and makes it possible to activate a wider range of genes than is possible using other technologies. For example, U.S. Patent No. 5,272,071 and foreign patent applications WO 91/06666, WO 91/06667 and WO 90/11354 describe homologous recombination methods for inserting a regulatory sequence upstream of the coding region of an endogenous gene. In these methods, only a very small number of positions for promoter insertion are acceptable for expression, limited by the frequent occurrence of ATG start codons as described above.

The present invention provides further advantages over the methods available in the art. For example, the use of homologous recombination results in the production of cells in which the novel transcription unit is present in the same location in all cells in which homologous recombination has occurred. Thus, the novel transcription unit will function similarly in all homologously recombinant cells derived independently. This allows for the production of cells with highly predictable properties. In the case of in vitro protein production, it is desirable to develop cells in which the behavior (e.g. the expression and amplification properties) of the desired gene can be controlled and there is little variation when comparing individual cells which are being processed for large-scale production purposes. In the case of in vivo protein production or gene therapy, it is desirable to be able to develop cells in which the properties are predictable and uniform among individual patients. This allows for a high degree of precision in achieving appropriate levels of the desired protein in vivo, leading to controlled and reproducible methods for treating disease.

The DNA constructs described above are useful for operatively linking exogenous regulatory and structural elements to endogenous coding sequences in a way that

-34-

precisely creates a novel transcriptional unit, provides flexibility in the relative positioning of exogenous regulatory elements and endogenous genes and, ultimately, enables a highly controlled system for and regulating
5 expression of genes of therapeutic interest.

The subject invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

10 EXAMPLE 1: Cloning of the TPO Gene and Identification of
5' Flanking Sequences

The human thrombopoietin gene was isolated from a human genomic DNA library. The library was prepared from male leukocyte DNA partially-digested with *Mbo*I and cloned
15 into the bacteriophage vector lambda EMBL3 (Clontech, Palo Alto, CA; Cat. #HL1006d). For screening, a probe was isolated by PCR amplification of human genomic DNA using oligonucleotides 1.1 and 1.2.

Oligo 1.1 (TPO sense) (SEQ ID NO: 1)

20 5' AATTGCTCCT CGTGGTCATG CTTCT

Oligo 1.2 (TPO anti-sense) (SEQ ID NO: 2)

5' CTGTGAAGGA CATGGGAGTC A

These primers were designed using the known TPO mRNA sequence (de Sauvage, F. J. et al. Nature 369:533-538
25 (1994)). The amplified probe (probe A; 120 bp) was labeled with ³²P dCTP by the polymerase chain reaction and used to screen the genomic DNA library. Filters were hybridized

-35-

for 6 hours at 68°C in 125 mM Na₂HPO₄ (pH 7.2), 250 mM NaCl, 10% PEG 8000, 7% SDS, 1 mM EDTA. Filters were washed twice in 500 ml of 20 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, 5% SDS, followed by 4 washes in 500 ml of 20 mM Na₂HPO₄ (pH 7.2), 1 mM EDTA, 1% SDS. The wash buffers were pre-heated to 56°C and washing was done on a rotary shaker at room temperature for approximately 5 minutes per wash. The hybridizing signals were identified by autoradiography at -80°C with an intensifying screen. In one experiment, approximately 1.4 x 10⁶ phage were screened and 7 positive signals were obtained. Phage plaques corresponding to positive signals were plaque purified. Following 2 rounds of plaque purification by low density screening using probe A, 4 of the phage, designated 5B, 25A, 25B and 28B, were retained for further analysis. Plaque purified phage were amplified and isolated by cesium chloride gradient ultracentrifugation (Yamamoto K.R. et al., *Virology* 40:734 (1970)) and DNA was isolated. Library screening, plaque purification of recombinant bacteriophage, and isolation of bacteriophage DNA was performed using standard methods (Ausubel et al., *Current Protocols in Molecular Biology*, Wiley, New York, NY. (1987)).

An approximately 6.9 kb XbaI fragment comprising exon 1, intron 1, exon 2, intron 2, exon 3, and a portion of intron 3, as well as approximately 4.3 kb of nontranscribed DNA lying upstream of TPO exon 1 was identified by restriction enzyme and Southern hybridization analysis using probe A. This fragment was isolated from one genomic clone (28B) and subcloned into plasmid pBSIISK⁺ (Stratagene Inc., La Jolla, CA) for further analysis. The resultant clones, pBS(X)/5'Thromb.8 and pBS(X)/5'Thromb.2, harbor the 6.9 kb XbaI fragment in opposite orientations with respect to the plasmid backbone. Restriction enzyme mapping yielded the restriction enzyme map shown in Figure 3. The nucleotide sequence of the portion of this fragment lying

-36-

upstream of the 5' end of the known cDNA sequence is shown in Figure 4 (SEQ ID NO: 3). The nucleotide sequence of the portion of the 6.9 kb *Xba*I fragment lying downstream of the 5' end of the known cDNA sequence is shown in Figure 5 (SEQ ID NO: 4). Comparison of the cloned genomic sequence presented here with the published cDNA sequence (de Sauvage, F. J. et al., *Nature* 369:533-538 (1994)) reveals that the 5' end of the TPO gene consists of a non-coding exon (exon 1) of at least 107 bp, a second exon (exon 2) which is 158 bp, and a third exon (exon 3) which is 128 bp in length. The 13 base pairs at the 3' end of exon 2 code for the first four and a portion of the fifth amino acid of the TPO signal peptide. Exon 3 codes for the remainder of the 21 amino acid signal peptide and a portion of the mature TPO polypeptide. Exons 1 and 2 are separated by intron 1 (1671 bp), and exons 2 and 3 are separated by intron 2 (231 bp). There are two differences between the sequence reported in Figure 5 and the sequence published by de Sauvage et al.: nucleotides at positions -134 and -124 are reported as C residues by de Sauvage et al. and are shown as T residues in Figure 5. These residues are outside of the coding sequence for TPO and may be explained by sequence polymorphism or by errors in compilation of the published sequence. In any event, this minor difference does not impact the ability of the person of skill to practice the invention as described herein.

EXAMPLE 2: Construction of Targeting Plasmids for
Activation and Amplification of the TPO Gene

The activation of the TPO gene can be accomplished by a number of strategies, as shown in Figures 6-8. In the strategy shown in Figure 6, a targeting fragment is introduced into the genome of recipient cells for insertion of a regulatory region, a non-coding exon, and a functional, unpaired splice-donor site upstream of the TPO

-37-

coding region. Specifically, the targeting construct from which this fragment is derived (pRTP01) is designed to include a first targeting sequence homologous to sequences upstream of the *TPO* gene, an amplifiable marker gene, a
5 selectable marker gene, a regulatory region, a CAP site, a non-coding exon, an unpaired splice-donor site, and a second targeting sequence corresponding to sequences downstream of the first targeting sequence but upstream of
10 *TPO* exon 1. By this strategy, homologously recombinant cells produce an mRNA precursor which includes the non-coding exon introduced upstream of the *TPO* gene by homologous recombination, the second targeting sequence and any sequences between the second targeting sequence and
15 exon 2 of the *TPO* gene, and the remaining exons, introns, and 3' untranslated regions of the *TPO* gene (Figure 6). Splicing of this message results in the fusion of the exogenous non-coding exon to exon 2 of the endogenous *TPO* gene which, when translated, will produce *TPO*. In this strategy the first and second targeting sequences are
20 upstream of the normal target gene, but this is not required (see below). The size of the intron in the targeting construct and thus the position of the regulatory region relative to the coding region of the gene may be varied to optimize the function of the regulatory region.

25 Plasmid pRTP01 is constructed as follows: Based on the restriction map of the *TPO* upstream region (Figure 3), a 3.5 kb *Bam*HI fragment can be isolated from subclone pBS(X)/5'Thromb.8 (Example 1). This fragment is ligated to *Bam*HI digested plasmid pBS (Stratagene, Inc., La Jolla, CA)
30 and transformed into competent *E. coli* cells to generate pBS-TPO1. This fragment includes sequences lying upstream of *TPO* exon 1. Next, a 0.73 kb fragment was amplified from hGH expression construct pXGH308, which has the CMV immediate-early (IE) gene promoter region beginning at
35 nucleotide 546 and ending at nucleotide 2105 of Genbank

-38-

sequence HS5MIEP fused to the hGH sequences beginning at nucleotide 5225 and ending at nucleotide 7322 of Genbank sequence HUMGHCSA, using oligonucleotides 2.1 and 2.2.

- (The source of the CMV IE gene is not critical, and other
- 5 CMV IE promoter-based plasmids may be used, or wild-type CMV DNA may be used.) Oligo 2.1 (37 bp, SEQ ID NO: 5), hybridizes to the CMV IE promoter at -614 relative to the cap site (in Genbank sequence HEHCMVP1), and includes a
- 10 NotI site followed by a partially overlapping XhoI site at its 5' end. Oligo 2.2 (36 bp, SEQ ID NO: 6), hybridizes to the CMV IE promoter at +131 relative to the cap site and includes the first 10 base pairs of the first intron of the CMV IE gene and contains a NotI site at its 5' end. The resulting PCR fragment is digested with NotI and
- 15 gel-purified. Plasmid pBS-TPO1 is digested with NotI, which cleaves at a single site upstream of TPO exon 1 (Figure 3), and the digested DNA is ligated to the CMV promoter fragment prepared above and transformed into competent *E. coli* cells. Colonies containing inserts of
- 20 the CMV promoter inserted at the NotI site of pBS-TPO1 are analyzed by restriction enzyme analysis to confirm the orientation of the insert, and one recombinant plasmid in which the CMV promoter is oriented such that the direction of transcription is towards TPO exon 1 is identified and
- 25 designated pBS-TPO2.

Oligo 2.1 (SEQ ID NO: 5)

5' TTTTGCGGCC GCTCGAGGAC ATTGATTATT GACTAGT
NotI XhoI

Oligo 2.2 (SEQ ID NO: 6)

30 5' TTTTGCGGCC GCCGGTACTT ACGTCACTCT TGGCAC
NotI

-39-

Next, the neomycin phosphotransferase (*neo*) gene is inserted into pBS-TPO2 for use as a selectable marker in isolating stably transfected human cells. Plasmid pMC1neoPolyA [Thomas, K.R. and Capecchi, M.R. Cell 51:503-512 (1987); available from Stratagene Inc., La Jolla, CA] is digested with *Bam*HI and made blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase. The treated DNA is then ligated to a double-stranded 10 base pair *Cla*I linker of the sequence 5'GGATCGATCC, chosen such that the *Bam*HI site is not regenerated by the linker addition. The resulting DNA is digested with *Cla*I and the digested DNA is ligated under dilute conditions to promote recircularization and transformed into competent *E. coli* cells. Transformed colonies are analyzed by restriction enzyme digestion to identify cells containing a derivative of plasmid pMC1neoPolyA with an insertion of a *Cla*I site at the 3' end of the *neo* gene. This plasmid is designated pMC1neo-C. pMC1neo-C is digested with *Xho*I and *Sal*I and the approximately 1.1 kb fragment containing the *neo* expression unit is gel purified. Plasmid pBS-TPO2 is digested at the unique *Xho*I site which was introduced by PCR at the 5' end of the CMV promoter, and the digested DNA is ligated to the purified *Xho*I-*Sal*I fragment containing the *neo* gene and transformed into competent *E. coli* cells. Colonies containing inserts of the *neo* gene inserted at the *Xho*I site of pBS-TPO2 are analyzed by restriction enzyme analysis to confirm the orientation of the insert, and one recombinant plasmid in which the *neo* gene is oriented such that the direction of transcription is opposite to CMV is identified and designated pBS-TPO3.

Finally, the targeting construct pTPO1 is constructed by insertion of a *dhfr* expression unit (to select for amplification in targeted human cells) at the *Cla*I site located at the 5' end of the *neo* gene of pBS-TPO3. To

-40-

obtain a *dhfr* expression unit, the plasmid construct pF8CIS9080 [Eaton et al., *Biochemistry* 25: 8343-8347 (1986)] is digested with *EcoRI* and *SalI*. A 2 kb fragment containing the *dhfr* expression unit is purified from this
5 digest and made blunt by treatment with the Klenow fragment of DNA polymerase I. A *ClaI* linker (New England Biolabs, Beverly, MA) is then ligated to the blunted *dhfr* fragment. The products of this ligation are digested with *ClaI* ligated to *ClaI* digested pBS-TPO3. An aliquot of this
10 ligation is transformed into *E. coli* and plated on ampicillin selection plates. Bacterial colonies are analyzed by restriction enzyme digestion to determine the orientation of the inserted *dhfr* fragment. One plasmid with *dhfr* in a transcriptional orientation opposite that of
15 the *neo* gene is designated pRTPO1. For targeting to the TPO locus in cultured human cells, pRTPO1 is digested with *BamHI* to separate the targeting fragment containing the targeting DNA, *neo* gene, *dhfr* gene, CMV promoter, and splice-donor site from the pBS plasmid backbone.
20 A second strategy for activation of the TPO gene is shown in Figure 7. In this strategy, a targeting fragment is introduced into the genome of recipient cells for insertion of a regulatory region, a non-coding exon, a splice-donor site, an intron, a splice-acceptor site, a
25 second non-coding exon, and a functional, unpaired splice-donor site upstream of the TPO coding region. Specifically, the targeting construct from which this fragment is derived (pRTPO2) is designed to include a first targeting sequence homologous to sequences upstream of the
30 TPO gene, an amplifiable marker gene, a selectable marker gene, a regulatory region, a CAP site, a non-coding exon, a splice-donor site, an intron, a splice-acceptor site, a second non-coding exon, an unpaired splice-donor site, and a second targeting sequence corresponding to sequences
35 downstream of the first targeting sequence but upstream of

-41-

TPO exon 2. By this strategy, homologously recombinant cells produce an mRNA precursor which corresponds to the first and second non-coding exogenous exons separated by an intron, the second targeting sequence, any sequences
5 between the second targeting sequence and exon 2 of the TPO gene, and the remaining exons, introns, and 3' untranslated regions of the TPO gene (Figure 7). Splicing of this message results in the fusion of the second non-coding
10 exogenous exon to exon 2 of the endogenous TPO gene which, when translated, will produce TPO. In this strategy the first and second targeting sequences are upstream of the normal target gene, but this is not required (see below). The size of the intron in the targeting construct and thus
15 the position of the regulatory region relative to the coding region of the gene may be varied to optimize the function of the regulatory region.

Plasmid pRTPO2 is constructed as follows: Based on the restriction map of the TPO upstream region (Figure 3), a 1.8 kb *Bam*HI-*Eco*RI fragment can be isolated from subclone
20 pBS(X)/5'Thromb.8 (Example 1). This fragment is ligated to *Bam*HI and *Eco*RI digested plasmid pBS (Stratagene, Inc., La Jolla, CA) and transformed into competent *E. coli* cells to generate pBS-TPO4. This fragment includes TPO exon 1 but contains no TPO coding sequences.

25 Next, oligonucleotides 2.3 to 2.6 are used in PCR to fuse CMV IE promoter sequences beginning at nucleotide 546 and ending at nucleotide 2105 of Genbank sequence HS5MIEP to sequences from the TPO gene comprised of exon 1 and a portion of intron 1. The properties of these primers are
30 as follows: 2.3 (SEQ ID NO: 7) is a 30 base oligonucleotide homologous to a segment of the CMV IE promoter beginning at nucleotide 546 of Genbank sequence HS5MIEP (-614 relative to the cap site) and includes a *Xho*I site at its 5' end; 2.4 (SEQ ID NO: 8) and 2.5 (SEQ ID NO:
35 9) are 60 nucleotide complementary primers which define the

-42-

fusion of CMV (position 2100 of Genbank sequence HS5MIEP) and TPO (position -1881 relative to the TPO translation start site) sequences; 2.6 (SEQ ID NO: 10) is 27 nucleotides in length and is homologous to TPO sequences ending in TPO intron 1 at position -1374 relative to the TPO translation start site and includes a natural *Apa*I site.

Oligo 2.3 (SEQ ID NO: 7)

10 5' TTTTCTCGAG GACATTGATT ATTGACTAGT
*Xho*I

Oligo 2.4 (SEQ ID NO: 8)

5' catgggtctt ttctgcagtc accgtccttg CTACCCATCT GCTCCCCAGA
GGGCTGCCTG

Oligo 2.5 (SEQ ID NO: 9)

15 5' CAGGCAGCCC TCTGGGGAGC AGATGGGTAG caaggacggt gactgcagaa
aagacccatg

Oligo 2.6 (SEQ ID NO: 10)

5' TTTGGGCCC TCCTCCCAT ACCCTCT
*Apa*I

20 Oligos 2.3-2.6: Bases in lower-case type denote CMV
sequences; bases in upper-case type denote TPO sequences

These primers are used to amplify a 2.1 kb DNA fragment comprising a fusion of CMV IE and TPO sequences. The fusion fragment is created by first using oligos 2.3

-43-

and 2.4 to amplify a 1.6 kb fragment from hGH expression construct pXGH308, which has the CMV immediate-early (IE) gene promoter region beginning at nucleotide 546 and ending at nucleotide 2105 of Genbank sequence HS5MIEP fused to the hGH sequences beginning at nucleotide 5225 and ending at nucleotide 7322 of Genbank sequence HUMGHCSA. (The source of the CMV IE gene is not critical, and other CMV IE promoter-based plasmids may be used, or wild-type CMV DNA may be used.) Then, oligos 2.5 and 2.6 are used to amplify a 0.54 kb fragment containing portions of TPO exon 1 and TPO intron 1 from plasmid pBS(X)/5'Thromb.8 (Example 1). The two amplified fragments are then combined and further amplified using oligos 2.3 and 2.6. The resulting product, a 2.1 kb PCR fragment is digested with *XhoI* and *ApaI* and gel purified. Plasmid pMCneo-C (see above) is digested with *SalI* and *XhoI* and the 1.1 kb neo containing fragment is gel purified. The purified 2.1 kb PCR fragment and the 1.1 kb neo fragment are then mixed and ligated to pBS-TPO4 (above) which has been cut with *SalI* and *ApaI*. The ligation mixture is transformed into *E. coli* cells and a plasmid with a single insert of each the fusion fragment and the neo gene is identified, this plasmid having the *SalI* site at the 3' end of the neo gene regenerated by ligation to the *SalI* site in the polylinker of pBS-TPO4. The resulting plasmid is designated pBS-TPO5.

A *dhfr* expression unit (to select for amplification in targeted human cells) is then inserted at the *ClaI* site located at the 5' end of the neo gene of pBS-TPO5. The *dhfr* expression unit is isolated from plasmid pF8CIS9080 [Eaton et al., *Biochemistry* 25: 8343-8347 (1986)] by digestion with *EcoRI* and *SalI*. A 2 kb fragment containing the *dhfr* expression unit is purified from this digest and made blunt by treatment with the Klenow fragment of DNA polymerase I. A *ClaI* linker (New England Biolabs, Beverly, MA) is then ligated to the blunted *dhfr* fragment. The

-44-

products of this ligation are digested with *Cla*I ligated to *Cla*I digested pBS-TPO5. An aliquot of this ligation is transformed into *E. coli* and plated on ampicillin selection plates. Bacterial colonies are analyzed by restriction enzyme digestion to determine the orientation of the inserted *dhfr* fragment. One plasmid with *dhfr* in a transcriptional orientation opposite that of the *neo* gene is designated pBS-TPO6.

To complete plasmid pRTPO2, plasmid pBS(X)/5'Thromb.8 (Example 1) is partially digested with *Bam*HI and ligated to a *Sal*I linker. The resulting DNA is then digested with *Sal*I and *Hind*III and the 3.7 kb fragment consisting of sequences upstream of the *TPO* gene is isolated for use as a second targeting sequence. This fragment is ligated to *Hind*III-*Sal*I digested pBS-TPO6 to generate the targeting plasmid pRTPO2. For targeting to the *TPO* locus in cultured human cells, pRTPO2 is digested with *Hind*III and *Eco*RI to separate the targeting fragment containing the targeting DNA, *neo* gene, *dhfr* gene, and CMV promoter from the pBS plasmid backbone.

A third strategy for activation of the *TPO* gene is shown in Figure 8. In this strategy, a targeting fragment is introduced into the genome of recipient cells for replacement of the normal *TPO* regulatory region, *TPO* exon 1, *TPO* intron 1, and *TPO* exon 2 with an exogenous regulatory region, a coding exon, and a functional, unpaired splice-donor site. Specifically, the targeting construct from which this fragment is derived (pRTPO3) is designed to include a first targeting sequence homologous to sequences upstream of the *TPO* gene, an amplifiable marker gene, a selectable marker gene, a regulatory region, a CAP site, an exon which includes sequences coding for the first 3 1/3 amino acids of the human growth hormone (hGH) signal peptide, an unpaired splice-donor site, and a second targeting sequence corresponding to *TPO* intron 2 sequences.

-45-

By this strategy, homologously recombinant cells produce an mRNA precursor which corresponds to the exogenous coding exon, intron 2 of the TPO gene, exon 3 of the TPO gene, and the remaining exons, introns, and 3' untranslated regions of the TPO gene (Figure 8). Splicing of this message results in the fusion of the exogenous coding exon to exon 3 of the endogenous TPO gene which, when translated, will produce a fusion protein in which the first 3 amino acids of the signal peptide are derived from hGH. The signal peptide of this molecule is cleaved off prior to secretion from a cell to produce mature TPO. In this strategy the first targeting sequence is upstream of the normal target gene, while the second targeting sequence is within the gene, between exons 2 and 3. The position of the first targeting sequence and the amount of upstream DNA replaced or deleted by the targeting event may be varied to optimize the function of the regulatory region.

Plasmid pRTPO3 is constructed as follows:
Oligonucleotides 2.8 to 2.11 are used in PCR to fuse CMV IE promoter sequences beginning at nucleotide 546 and ending at nucleotide 1258 of Genbank sequence HS5MIEP to sequences from the human growth hormone gene which encode the first 3 1/3 amino acids of the hGH signal peptide, a splice donor site, and the second intron of the TPO gene. The properties of these primers are as follows: Oligo 2.8 (SEQ ID NO: 11) is a 30 base oligonucleotide homologous to a segment of the CMV IE promoter beginning at nucleotide 546 of Genbank sequence HS5MIEP (-614 relative to the cap site) and includes an XhoI site at its 5' end; 2.9 (SEQ ID NO: 12) and 2.10 (SEQ ID NO: 13) are 69 nucleotide complementary primers which define the fusion of CMV (position 2100 of Genbank sequence HS5MIEP) and hGH sequences (position -10 relative to the translation start site of the hGH gene; see the hGH gene N sequence in Genbank entry HUMGHCSA) sequences. These primers also

-46-

include the first 29 base pairs of TPO intron 2 (nucleotides +14 to +42 relative to the TPO translation start site), which include the splice donor site; 2.11 (SEQ ID NO: 14) is 45 nucleotides in length and is homologous to 5 TPO sequences in TPO intron 2 starting at position +182 relative to the TPO translation start site and extending upstream, and includes a natural EcoRI site at its 5' end.

The fusion fragment is created by first using oligos 2.8 and 2.9 to amplify a 0.7 kb fragment from CMV viral DNA 10 containing a wild-type immediate early gene and promoter sequence. (The source of the CMV IE gene is not critical, and other CMV IE promoter-based plasmids may be used.) Then, oligos 2.10 and 2.11 are used to amplify a 0.17 kb fragment containing a portion of TPO intron 2 from plasmid 15 pBS(X)/5'Thromb.8 (Example 1). The two amplified fragments are then combined and further amplified using oligos 2.8 and 2.11. The resulting product, a 0.9 kb PCR fragment is digested with XhoI and EcoRI and gel purified. Next, plasmid a pBS(X)/5'Thromb.8 (Example 1) is partially 20 digested with BamHI and ligated to an XhoI linker. The resulting DNA is then digested with XhoI and HindIII and the 3.9 kb fragment consisting of sequences upstream of the TPO gene is isolated for use as a second targeting sequence. This fragment contains sequences from -5985 to 25 -2095 relative to the TPO translation start site (Figure 3). The isolated fragment is then ligated in a mixture containing the 0.9 kb fusion fragment purified above and HindIII and EcoRI digested plasmid pBS (Stratagene, Inc., La Jolla, CA) and transformed into competent *E. coli* cells 30 to generate pBS-TPO7.

For insertion of the neo selectable marker gene, plasmid pMC1neo-C (see above) is digested with XhoI and SalI and ligated to XhoI digested pBS-TPO7. The ligation mix is transformed into *E. coli* cells and colonies are 35 analyzed by restriction enzyme analysis to identify a

-47-

plasmid with a single insert of the *neo* gene oriented such that the direction of transcription is opposite to that of the CMV promoter. This plasmid is designated pBS-TPO8.

5 A *dhfr* expression unit (to select for amplification in targeted human cells) is then inserted at the *Cla*I site located at the 5' end of the *neo* gene of pBS-TPO8. The *dhfr* expression unit is isolated from plasmid pF8CIS9080 [Eaton et al., *Biochemistry* 25: 8343-8347 (1986)] by digestion with *Eco*RI and *Sal*I. A 2 kb fragment containing 10 the *dhfr* expression unit is purified from this digest and made blunt by treatment with the Klenow fragment of DNA polymerase I. A *Cla*I linker (New England Biolabs, Beverly, MA) is then ligated to the blunted *dhfr* fragment. The products of this ligation are digested with *Cla*I ligated to 15 *Cla*I digested pBS-TPO8. An aliquot of this ligation is transformed into *E. coli* and plated on ampicillin selection plates. Bacterial colonies are analyzed by restriction enzyme digestion to determine the orientation of the inserted *dhfr* fragment. One plasmid with *dhfr* in a 20 transcriptional orientation opposite that of the *neo* gene is designated pRTPO3. For targeting to the TPO locus in cultured human cells, pRTPO3 is digested with *Eco*RI and *Hind*III to separate the targeting fragment containing the targeting DNA, *neo* gene, *dhfr* gene, CMV promoter, and hGH 25 coding DNA from the pBS plasmid backbone.

Oligo 2.8 (SEQ ID NO: 11)

5' TTTTCTCGAG GACATTGATT ATTGACTAGT

*Xho*I

Oligo 2.9 (SEQ ID NO: 12)

30 5' cgcggtattcc ccgtgccaaag CCTAGCGGCA ATGGCTACAG GTGAGAACAC
ACCTGAGGGG CTAGGGCCA

-48-

Oligo 2.10 (SEQ ID NO: 13)

5' TGGCCCTAGC CCCTCAGGTG TGTTCTCACC TGTAGCCATT GCCGCTAGGc
ttggcacggg gaatccgcg

Oligo 2.11 (SEQ ID NO: 14)

5' TTTGAATTC CCATTCAGGA CCCAGACCTG AAACCCAGGG AATCC
EcoRI

Oligos 2.8-2.11: Bases in lower-case type denote CMV sequences; upper-case, non-bold bases denote TPO sequences; boldface bases denote hGH exon 1 sequences.

- 10 Other approaches for targeting and activation of the TPO gene may be employed. For example, the first and second targeting sequences may correspond to sequences in the first or second intron of the TPO gene, and the targeting sequences may include TPO coding sequences. In
15 any activation strategy, the second targeting sequence does not need to lie immediately adjacent to or near the first targeting sequence in the normal gene, such that portions of the gene's normal upstream region are deleted upon homologous recombination. Furthermore, one targeting
20 sequence may be upstream of the gene and one may be within an exon or intron of the TPO gene.

- A selectable marker gene is optional and the amplifiable marker gene is only required when amplification is desired. The amplifiable marker gene and selectable
25 marker gene may be the same gene, their positions may be reversed, and one or both may be situated in the intron of the targeting construct. Amplifiable marker genes and selectable marker genes suitable for selection are described herein. The incorporation of a specific CAP site
30 is optional. The regulatory region, CAP site, first non-coding exon, splice-donor site, intron, second non-coding exon, and splice acceptor site may be isolated

-49-

as a complete unit from the human elongation factor-1a (EF-1a; Genbank sequence HUMEF1A) gene or the cytomegalovirus (CMV; Genbank sequence HEHCMVP1) immediate early region, or the components can be assembled from appropriate components isolated from different genes. In any case, either exogenous exon may be the same or different from the first exon of the normal TPO gene, and multiple non-coding exons may be present in the targeting construct.

As described herein, a number of selectable and amplifiable markers may be used in the targeting constructs, and the activation may be effected in a large number of cell-types.

EXAMPLE 3: In Vitro Production of TPO by Activation and Amplification of the TPO Gene in an Immortalized Cell Line

Transfection of primary, secondary, or immortalized human cells and isolation of homologously recombinant cells expressing TPO may be accomplished using the methods described in U.S. Serial No. 08/243,391 incorporated by reference. Homologously recombinant cells may be identified by PCR screening strategy as exemplified therein and in published methods available to one skilled in the art (see, for example, Kim, H-S and Smithies, O., Nucl. Acids Res. 16:8887-8903 (1988)). The identification of cells expressing TPO may also be accomplished using a variety of assays based on the structure or properties of TPO. For example, TPO may be functionally identified by an in vitro or in vivo megakaryocytopoiesis assay (de Sauvage et al., Nature 369:533-538 (1994)). Alternatively, TPO may be assayed by the stimulation of proliferation of cells expressing the c-mpl ligand, the receptor for TPO. In this assay, cells such as Ba/F3-mpl cells (de Sauvage et al., Nature 369:533-538 (1994)), are exposed to TPO and cell

-50-

proliferation is monitored by ^3H -thymidine uptake. TPO may also be assayed through its effects on in vivo platelet production, either by direct platelet counts or by incorporation of ^{35}S into platelets. Finally, peptides
5 corresponding to portions of the TPO molecule may be synthesized in order to generate anti-TPO antibodies for use in an ELISA assay.

The isolation of cells containing amplified copies of the amplifiable marker gene and the activated TPO locus is
10 performed as described in U.S. Serial No.: 07/985,586 incorporated by reference.

EXAMPLE 4: Cloning of the Human DNase I Gene and
Identification of the 5' Flanking Sequences

The human DNase I gene was isolated from a human
15 genomic DNA library. The library (Clontech, Palo Alto, CA; Cat. #HL1006d) was constructed by cloning *Mbo*I partially digested male leukocyte DNA into the *Bam*HI site of the bacteriophage lambda vector EMBL3. For library screening, a DNA probe was isolated by PCR amplification of human
20 genomic DNA using oligonucleotides 4.1 and 4.2.

Oligo 4.1 (SEQ ID NO: 15)

5' TGCCTTGAAG TGCTTCTTCA

Oligo 4.2 (SEQ ID NO: 16)

5' CCTCAGAGAT GACGAGAATG C

25 These primers were designed based on the published DNase I mRNA sequence (Shak S. et al., Proc. Natl. Acad. Sci. USA 87:9188-9192 (1990)). The amplified probe (probe A; 126 bp) was labeled with ^{32}P -dCTP by PCR and used to

-51-

screen a bacteriophage lambda genomic DNA library. The filters were hybridized for 16 hours at 68°C in 125 mM Na₂HPO₄ (pH 7.2), 250 mM NaCl, 10% PEG 8000, 7% SDS, 1 mM EDTA. Filters were washed two times in 500 ml of 20 mM Na₂HPO₄ (pH 7.2), 5% SDS, 1 mM EDTA, followed by 4 washes in 500 ml of 20 mM Na₂HPO₄ (pH 7.2), 1% SDS, 1 mM EDTA. The wash buffers were preheated to 56°C and washing was performed at room temperature on a rotary shaker for approximately 5 minutes per wash. The hybridization signals were visualized by autoradiography at -80°C with an intensifying screen. In this experiment, approximately 1 x 10⁶ phage were screened and 18 positive signals were obtained. Bacteriophage plaques corresponding to 10 of the positive signals were plated at low density and subjected to a second round of screening using probe A. Four of the phage (designated 2a, 3b, 4c and 14a) gave positive hybridization signals following the secondary screening and were retained for further analysis. DNA was isolated from the plaque purified phage following amplification and subsequent purification by cesium chloride gradient ultracentrifugation (Yamamoto, K.R. et al., *Virology* 40:734 (1970)). Library screening, plaque purification of recombinant bacteriophage and isolation of bacteriophage DNA was performed using standard methods (Ausubel et al., *Current Protocols in Molecular Biology*. Wiley, New York, NY (1987)).

Based on restriction enzyme digestion and Southern blot analysis using probe A, two of the phage (4c and 14a) contain a common *HincII* fragment of approximately 8 kb which encompasses exon 1, intron 1, exon 2, coding and non-coding sequences corresponding to intron 2 and downstream *DNase I* exons, as well as approximately 4 kb of non-transcribed DNA lying upstream of *DNase I* exon I. This fragment was isolated from one genomic clone (4c) and subcloned into pBSIISK⁺ (Stratagene Inc., La Jolla, CA) for

-52-

further analysis. Restriction enzyme mapping of the resultant clone, pBS/ 4C.2Hinc2, was used to generate the restriction map shown in Figure 9. The nucleotide sequence of the non-transcribed *DNase I* 5' region lying upstream of the 5' end of the known cDNA sequence is shown in Figure 10 (SEQ ID NO: 17). The nucleotide sequence lying downstream of the 5' end of the known cDNA sequence, including exon 1, intron 1 and part of exon 2 is shown in Figure 11 (SEQ ID NO: 18). Comparison of the cloned genomic sequence presented here, with the published cDNA sequence (Shak, S. et al., *Proc. Natl. Acad. Sci. USA* 87:9188-9192 (1990)) reveals that the 5' end of the *DNase I* gene consists of a non-coding exon (exon 1) of 142 bp and a second exon (exon 2) which is at least 341 bp. Exon 2 encodes a 22 amino acid signal sequence and a portion of the mature *DNase I* peptide, beginning with an AUG translational initiation codon which lies 1 bp downstream of the 5' end of exon 2. Exons 1 and 2 are separated by intron 1 which is 336 bp in length.

20 EXAMPLE 5: Construction of Targeting Plasmids for
Activation and Amplification of the *DNase I*
Gene

The activation of the *DNase I* gene can be accomplished by the strategy outlined in Figure 12. In this strategy, a targeting fragment is introduced into the genome of recipient cells for insertion of a regulatory region, a non-coding exon and a functional unpaired splice-donor site upstream of the *DNase I* coding region. Specifically, the targeting construct from which this fragment is derived (pDNase1), is designed to include a 5' targeting sequence homologous to sequences upstream of the *DNase I* gene, a selectable marker gene, an amplifiable marker gene, a regulatory region, a CAP site, a non-coding exon, an unpaired splice-donor site, and a 3' targeting sequence

-53-

corresponding to sequences downstream of the 5' targeting sequence but upstream of *DNase I* exon 1. According to this strategy, integration of the targeting construct by homologous recombination generates recombinant cells producing an mRNA precursor which includes the non-coding exon introduced upstream of the *DNase I* gene, the 3' targeting sequence, any sequences between the 3' targeting sequence and exon 2 of the *DNase I* gene, and the remaining exons, introns and 3' untranslated regions of the *DNase I* gene (Figure 12). Splicing of this transcript results in the fusion of the exogenous non-coding exon to exon 2 of the endogenous *DNase I* gene. *DNase I* is produced by translation of the mature mRNA. According to this strategy, both the 5' and 3' targeting sequences are upstream of the endogenous target gene. The size of the chimeric intron in the targeting construct, which is dictated by the position of the regulatory region relative to the coding sequence, may be varied to optimize the function of the regulatory region.

Plasmid pCND1, which contains the activation cassette, is constructed as follows: A 1555 bp (size includes a 9 bp synthetic *HindIII* recognition site at the 5' end of oligo 5.2) fragment is amplified using oligos 5.1 and 5.2. The amplified fragment encompasses the CMV IE promoter, CMV IE exon 1 (non-coding exon) and 827 bp of CMV IE intron 1, beginning at nucleotide 172,783 and ending at nucleotide 174,328 of EMBL sequence X17403 ((Human cytomegalovirus strain AD169). (The source of the CMV IE gene is not critical, and CMV IE promoter-based plasmids or wild-type CMV DNA may be used.) Oligo 5.1 (21 bp, SEQ ID NO: 19) hybridizes to the CMV IE promoter at -598 relative to the CAP site (EMBL sequence X17403). Oligo 5.2 (32 bp, SEQ ID NO: 20) contains 23 nucleotides which hybridize to the CMV IE promoter at +946 relative to the CAP site, the additional 9 bp at the 5' end of the oligo create a

-54-

synthetic *Hind*III recognition sequence. The 1555 bp PCR product is digested with *Hind*III and the resultant 1551 bp fragment is purified and used in the ligation described below. Next, the neomycin phosphotransferase (*neo*) gene is

5 isolated from plasmid pBSneo for use as a selectable marker for the isolation of stably transfected human cells. The *neo* gene in plasmid pBSneo was obtained by *Bam*HI and *Xho*I digestion of pMC1neo-polyA (Thomas, K.R. and Capecchi, M.R. Cell 51:503-512 (1987)). Plasmid pMC1neo-polyA was

10 digested with *Bam*HI and made blunt ended with the Klenow fragment of *E. coli* DNA polymerase I. The resulting DNA was digested with *Xho*I, and the blunt-ended *Bam*HI-*Xho*I fragment was cloned into *Hinc*II and *Xho*I digested plasmid pBSIISK⁺. For isolation of the *neo* gene harbored on

15 pBSneo, plasmid pBSneo is digested with *Xho*I and made blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase I. The resulting DNA is digested with *Hind*III and an 1165 bp fragment containing the *neo* expression unit is gel purified. The 1165 bp *neo* fragment

20 and the 1551 bp CMV promoter fragment are ligated, the ligation products are digested with *Hind*III and the 2716 bp *Hind*III fragment, resulting from blunt-end ligation of the two fragments, is gel purified. The 2716 bp *Hind*III product is ligated to *Hind*III digested plasmid pBSIISK⁺

25 (Stratagene Inc., La Jolla, CA) and electroporated into *E. coli*. Colonies containing inserts in the *Hind*III site of pBSIISK⁺ are analyzed by restriction enzyme analysis to confirm the orientation of the insert. One recombinant plasmid in which the CMV promoter is oriented such that the

30 oligo 5.2 sequences (+946 relative to the CMV IE CAP site) are proximal to the *Sal*I recognition sequence in the pBSIISK⁺ polylinker, is identified and designated pCN1.

Oligo 5.1 (SEQ ID NO: 19)

5' GACATTGATT ATTGACTAGT T

-55-

Oligo 5.2 (SEQ ID NO: 20)

5' TTTAAGCTTC TGCAGAAAAG ACCCATGGAA AG

Next, the *dhfr* expression unit is inserted at a *Cla*I site which is located at the 3' end of the *neo* gene of pCN1. The *dhfr* expression unit is obtained by *Eco*RI and *Sal*I digestion of plasmid pF8CIS9080 (Eaton et al., *Biochemistry* 25:8343-8347 (1986)). The resultant 2 kb fragment is purified from the digest and made blunt with the Klenow fragment of *E. coli* DNA polymerase I. A *Cla*I linker (5' CCATCGATGG (NEB 1088; New England Biolabs, Beverly, MA) is ligated to the blunt-end *dhfr* fragment and the ligation products are digested with *Cla*I. pCN1 is digested with *Cla*I, and the *Cla*I *dhfr* containing fragment is ligated into *Cla*I site of pCN1. An aliquot of the ligation reaction is electroporated into *E. coli* and colonies harboring inserts in a *Cla*I site of pCN1 are analyzed by restriction enzyme analysis to determine the site of insertion and the orientation of the insert. A plasmid with the *dhfr* expression unit at the 3' end of the *neo* gene and with the same transcriptional orientation as that of the *neo* gene is identified and designated pCND1.

Plasmid pDNase1 is constructed as follows: Based on the restriction map of the upstream region of the *DNase I* gene (Figure 9), a 664 bp *Bam*HI fragment (-1161 to -498 in figure 8) can be isolated from subclone pBS/4C.2Hinc2. This fragment is ligated to *Bam*HI digested plasmid pBSIISK*dApaI (modification of pBSIISK⁺; Stratagene Inc., La Jolla, CA) in which the *Apa*I recognition sequence in the polylinker is destroyed. pBSIISK*dApaI is constructed by digesting pBSIISK⁺ with *Apa*I, conversion of the cohesive-ends to blunt-ends with T4 DNA polymerase and ligation to generate the circular plasmid. Following ligation of the 664 bp *Bam*HI fragment into pBSIISK*dApaI, the ligation products are electroporated into *E. coli* cells

-56-

to generate pBS-DNase1. The sequences contained in this fragment reside upstream of *DNase I* exon 1, position -1162 to -498 with respect to the AUG translational initiation codon (nucleotide +1). The activation cassette which
5 contains the CMV immediate-early (IE) promoter region, the CMV IE CAP site, a non-coding exon, an unpaired splice donor site, the neomycin phosphotransferase (*neo*) selectable marker gene and *dhfr* expression unit (to select for amplification in targeted human cells) is cloned into
10 the unique *ApaI* site of the 664 bp *BamHI* fragment (*DNase I* upstream region) in pBS-DNase1 (see Figure 12). Specifically, plasmid pCND1 which contains the activation cassette, is digested with *SalI* which cuts downstream of the *dhfr* expression unit and *EspI* which cuts 242 bp
15 downstream of the CMV IE CAP site. A 3,955 bp *SalI-EspI* fragment containing the activation cassette is purified from this digest and the cohesive-ends are made blunt by treatment with the Klenow fragment of *E. coli* DNA polymerase I. This fragment is ligated to plasmid
20 pBS-DNase1, which has been digested with *ApaI* and made blunt-ended by treatment with T4 DNA polymerase I, and electroporated into *E. coli*. Colonies containing inserts of the activation cassette inserted at the blunt-ended *ApaI* site of pBS-DNase 1 are analyzed by restriction enzyme
25 analysis to confirm the orientation of the insert. One recombinant plasmid in which the CMV promoter is oriented such that the direction of transcription is towards *DNase I* exon 1 is identified and designated pDNase1.

Plasmid pDNase1 is digested with *BamHI* for
30 transfection into human cells. Transfection of primary, secondary, or immortalized human cells and isolation of homologously recombinant cells expressing *DNase I* may be accomplished using the methods described in U.S. Serial No. 08/243,391 and incorporated herein by reference.
35 Homologously recombinant cells may be identified by PCR

-57-

- screening strategy as exemplified therein and in published methods available to one skilled in the art (see, for example, Kim, H-S and Smithies, O., *Nucl. Acids Res.* 16:8887-8903 (1988)). The identification of cells
- 5 expressing DNase I may also be accomplished using a variety of assays based on the structure or properties of DNase I. For example, DNase I may be functionally identified by an in vitro enzyme assay (cf. Kunitz, *J. Gen. Physiol.* 33: 349 (1950); McDonald, *Meth. Enzymol.* 2:437 (1955)) or by the
- 10 use of anti-DNase I antibodies in an ELISA assay.

The isolation of cells containing amplified copies of the amplifiable marker gene and the activated DNase I locus is performed as described in U.S. Serial No.: 07/985,586 incorporated herein by reference.

15 EXAMPLE 6: Cloning of the Human β -Interferon Gene and Identification of the 5' Flanking Sequences

- The human β -interferon gene was isolated from a human genomic DNA library. The library (Clontech, Palo Alto, CA; Cat. #HL1006d) was constructed by cloning *Mbo*I partially
- 20 digested male leukocyte DNA into the *Bam*HI site of the bacteriophage lambda vector EMBL3. For library screening, a DNA probe was isolated by PCR amplification of human genomic DNA using oligonucleotides 6.1 and 6.2

Oligo 6.1 (SEQ ID NO: 21)

- 25 5' TGCTCTGGCA CAACAGGTAG

Oligo 6.2 (SEQ ID NO: 22)

5' CATAGATGGT CAATGCGGC

- These primers were designed based on the published β -interferon mRNA sequence (May, L.T. and Sehgal, P.B., *J. Interferon Res.* 5:521-526 (1985)). The amplified probe
- 30

-58-

(probe A; 290 bp) was labeled with ^{32}P -dCTP by PCR and used to screen a bacteriophage lambda genomic DNA library. The filters were hybridized for 16 hours at 68°C in 125 mM Na_2HPO_4 (pH 7.2), 250 mM NaCl, 10% PEG 8000, 7% SDS, 1 mM EDTA. Filters were washed two times in 500 ml of 20 mM Na_2HPO_4 (pH 7.2), 5% SDS, 1 mM EDTA, followed by 4 washes in 500 ml of 20 mM Na_2HPO_4 (pH 7.2), 1% SDS, 1 mM EDTA. The wash buffers were preheated to 56°C and washing was performed at room temperature on a rotary shaker for approximately 5 minutes per wash. The hybridization signals were visualized by autoradiography at -80°C with an intensifying screen. In this experiment, approximately 1×10^6 phage were screened and 6 positive signals were obtained. Bacteriophage plaques corresponding to the positive signals were plated at low density and subjected to a second round of screening using probe A. Five of the phage (designated 1a, 2a, 2b, 11a, and 12a) gave positive hybridization signals following the secondary screening and were retained for further analysis. DNA was isolated from the plaque purified phage following amplification and subsequent purification by cesium chloride gradient ultracentrifugation (Yamamoto, K.R. et al., *Virology* 40:734 (1970)). Library screening, plaque purification of recombinant bacteriophage and isolation of bacteriophage DNA was performed using standard methods (Ausubel et al., *Current Protocols in Molecular Biology*. Wiley, New York, NY (1987)).

Based on restriction enzyme digestion and Southern blot analysis using probe A, all five of the phage (1a, 2a, 2b, 11a, and 12a) were shown to contain a common *HindIII* fragment of approximately 10 kb which encompasses the entire sequence coding for β -interferon (561 bp), 666 bp of 3' untranslated sequence and approximately 9 kb of non-transcribed DNA lying upstream of the β -interferon gene. This fragment was isolated from one genomic clone

-59-

(1a) and subcloned into pBSIISK⁺ (Stratagene Inc., La Jolla, CA) for further analysis. The resultant clones, pBS-H3/Bint.11-3 and pBS-H3/Bint.11-21, harbor the 10 kb HindIII fragment in opposite orientations with respect to the plasmid backbone. Restriction enzyme mapping was used to generate the restriction map shown in Figure 13. The nucleotide sequence of 8,355 bp of DNA lying upstream of the previously reported sequence (Genbank entry HUMIFNB1F) is shown in Figure 14 (SEQ ID NO: 23). The nucleotide sequence corresponding to 356 bp of DNA upstream of the β -interferon coding region, the β -interferon coding region, and 666 bp of 3' untranslated sequence is shown in Figure 15 (SEQ ID NO: 24). Comparison of the cloned genomic sequence presented here, with the published cDNA sequence (May, L.T. and Sehgal, P.B., *J. Interferon Res.* 5:521-526 (1985)) confirms that the β -interferon gene consists of a 561 bp coding region which is co-linear with its cognate mRNA (lacks introns). The β -interferon gene encodes a 21 amino acid signal sequence and a 120 amino acid mature peptide, beginning with an AUG translational initiation codon which lies 82 bp downstream of the CAP site.

EXAMPLE 7: Construction of Targeting Plasmids for Activation and Amplification of the β -Interferon Gene

The activation of the β -interferon gene can be accomplished by the strategy outlined in Figure 16. In this strategy, a targeting fragment is introduced into the genome of recipient cells for replacement of the endogenous β -interferon regulatory region with an exogenous regulatory region, a non-coding exon, an intron, and chimeric exon sequences consisting of sequences from a noncoding exon (derived from exon 2 of the CMV IE gene) and sequences from the β -interferon 5' noncoding region. Specifically, the targeting construct from which this fragment is derived

-60-

(pIFN β -1) is designed to include a 5' targeting sequence homologous to sequences upstream of the β -interferon gene, a selectable marker gene, an amplifiable marker gene, a regulatory region, a CAP site, a non-coding exon, an intron, chimeric exon sequences consisting of CMV IE exon 2 sequences and β -interferon 5' noncoding DNA, and a 3' targeting sequence homologous to DNA upstream of the β -interferon coding region. According to this strategy, integration of the targeting construct by homologous recombination generates recombinant cells producing an mRNA precursor which includes the non-coding exon introduced upstream of the β -interferon gene, an intron, the chimeric exon which fuses CMV IE exon sequences to β -interferon 5' noncoding sequences and the entire β -interferon coding region, and 3' untranslated regions of the β -interferon gene (Figure 16). The chimeric exon consists of 17 bp of CMV IE exon 2 (position 172,782 to 172,766 of EMBL sequence X17403) joined to the 5' flanking region of the β -interferon gene (position -173 with respect to the AUG translational initiation codon). Splicing of this transcript results in the fusion of the exogenous non-coding exon to exon 2 which includes the complete coding sequence of the endogenous β -interferon gene. β -interferon is produced by translation of the mature mRNA. According to this strategy, the 5' targeting sequence is upstream of the endogenous target gene and the 3' targeting sequence is in the β -interferon 5' noncoding region. The position of the regulatory region relative to the 5' flanking sequence, may be varied (e.g. by altering the size of the intron in the targeting construct) to optimize the function of the regulatory region.

Plasmid pIFN β -1 is constructed as follows: A 182 bp fragment (size includes a 9 bp synthetic *Bam*HI recognition site at the 5' end of Oligo 7.1) is amplified from pBS-H3/Bint.11-3 using oligos 7.1 and 7.2. The amplified

-61-

fragment serves as the 3' targeting sequence (Figure 16). Oligo 7.1 (21 bp, SEQ ID NO: 25) hybridizes to the β -interferon 5' non-transcribed region at position -173 with respect to the β -interferon AUG translational initiation codon (Figure 15). Oligo 7.2 (30 bp, SEQ ID NO: 26) contains 21 nucleotides which hybridize to the β -interferon 5' untranslated region at position -1 relative to the AUG translational start codon (see Figure 16), with the additional 9 bp at the 5' end of the oligo creating a synthetic *Bam*HI recognition sequence. The 182 bp PCR product is purified and used in the ligation described below. Next, a 1571 bp (size includes an 8 bp synthetic *Sma*I recognition sequence at the 5' end of oligo 7.3) fragment is amplified using oligos 7.3 and 7.4. The amplified fragment encompasses the CMV IE promoter, CMV IE exon 1 (non-coding exon), CMV IE intron 1 and 17 bp of CMV IE exon 2, beginning at nucleotide 174,328 and ending at nucleotide 172,766 of EMBL sequence X17403 (Human cytomegalovirus strain AD 169). (The source of the CMV IE gene is not critical, and CMV IE promoter-based plasmids or wild type CMV DNA may be used). Oligo 7.3 (29 bp, SEQ ID NO: 27) contains 21 nucleotides which hybridize to the CMV IE promoter at -598 relative to the CAP site (EMBL sequence X17403), the 5' end of the oligo also contains a 8 bp synthetic *Sma*I recognition sequence. Oligo 7.4 (21 bp, SEQ ID NO: 28) hybridizes to the CMV IE promoter at +965 relative to the CAP site. The 1571 bp PCR product containing the CMV IE promoter, CMV IE exon 1, CMV IE intron 1 and 23 bp of CMV IE exon 2, is gel purified and ligated to the 182 bp fragment containing the β -interferon 5' flanking region. The ligation products are digested with *Bam*HI and *Sma*I, and the 1742 bp *Sma*I-*Bam*HI fragment, resulting from ligation of β -interferon sequences (position -173 with respect to the AUG translational initiation codon) to CMV IE sequences (-598 relative to the CMV IE CAP

-62-

site), is gel purified. The 1742 bp *Sma*I-*Bam*HI fragment is ligated to *Bam*HI and *Sma*I digested plasmid pBSIISK⁺ (Stratagene Inc., La Jolla, CA) and electroporated into *E. coli*. Colonies containing inserts in pBSIISK⁺ are analyzed
5 by restriction enzyme analysis to confirm the structure of the insert. One recombinant plasmid is identified and designated pBS-CB.

Oligo 7.1 (SEQ ID NO: 25)

5' TGACATAGGA AACTGAAAG G

10 Oligo 7.2 (SEQ ID NO: 26)

5' TTTGGATCCG TTGACAACAC GAACAGTGTC G

Oligo 7.3 (SEQ ID NO: 27)

5' TTTCCCGGGA CATTGATTAT TGACTAGTT

Oligo 7.4 (SEQ ID NO: 28)

15 5' CGTGTCAAGG ACGGTGACTG C

The neomycin phosphotransferase (*neo*) gene is isolated from plasmid pBSneo for use as a selectable marker for the isolation of stably transfected human cells. The *neo* gene in plasmid pBSneo was obtained by *Bam*HI and *Xho*I digestion
20 of pMC1neo-polyA (Thomas, K.R. and Capecchi, M.R., Cell 51:503-512 (1987)). Plasmid pMC1neo-polyA was digested with *Bam*HI and made blunt ended with the Klenow fragment of *E. coli* DNA polymerase I. The resulting DNA was digested with *Xho*I, and the blunt-ended *Bam*HI-*Xho*I fragment was
25 cloned into *Hinc*II and *Xho*I digested plasmid pBSIISK⁺. For isolation of the *neo* gene harbored on pBSneo, plasmid pBSneo is digested with *Xho*I and made blunt-ended by treatment with the Klenow fragment of *E. coli* DNA polymerase I. The resulting DNA is digested with *Hind*III
30 and a 1165 bp fragment containing the *neo* expression unit

-63-

is gel purified. The 1165 bp fragment is ligated to *Sma*I and *Hind*III digested plasmid pBS-CB and electroporated into *E. coli*. Colonies containing inserts in pBS-CB are analyzed by restriction enzyme analysis to confirm the orientation of the insert. One recombinant plasmid is identified and designated pBS-CBN.

Next, the *dhfr* expression unit is inserted at the *Cla*I site which is located at the 3' end of the *neo* gene of pBS-CBN. The *dhfr* expression unit is obtained by *Eco*RI and *Sal*I digestion of plasmid pF8CIS9080 (Eaton et al., *Biochemistry* 25:8343-8347 (1986)). The resultant 2 kb fragment is purified from the digest and made blunt with the Klenow fragment of *E. coli* DNA polymerase I. A *Cla*I linker (5' CCATCGATGG; NEB 1088, New England Biolabs, Beverly, MA) is ligated to the blunt-end *dhfr* fragment, the ligation products are digested with *Cla*I and purified. The *Cla*I *dhfr* containing fragment is ligated into *Cla*I digested plasmid pBS-CBN. An aliquot of the ligation reaction is electroporated into *E. coli* and colonies harboring inserts in a *Cla*I site of pBS-CBN are analyzed by restriction enzyme analysis to determine the site of insertion and the orientation of the insert. A plasmid with the *dhfr* expression unit at the 3' end of the *neo* gene and with the same transcriptional orientation as that of the *neo* gene is identified and designated pBS-CBND.

Finally, the targeting construct is constructed by insertion of the 5' targeting sequence (Figure 16) in the unique *Sal*I site located at the 3' end of the *dhfr* expression unit in plasmid pBS-CBND. To obtain the 5' targeting sequence, the plasmid pBS-H3/Bint.11-3 is digested with *Eco*RI and *Pvu*II and the resultant 1.2 kb fragment is purified, ligated to *Eco*RI-*Sma*I digested plasmid pBSIISK⁺ (Stratagene Inc., La, Jolla, CA) and electroporated into *E. coli*. Colonies containing inserts in pBSIISK⁺ are analyzed by restriction enzyme analysis,

-64-

and one plasmid containing the insert is retained and designated pBS-BI5. Plasmid pBS-BI5 is digested with *SpeI* and *EcoRV* and made blunt-ended with the Klenow fragment of DNA polymerase I. The resulting 1.2 kb fragment is ligated
5 to *SalI* digested plasmid pBS-CBND, which has been made blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I. An aliquot of the blunt-end ligation reaction is electroporated into *E. coli* and colonies harboring inserts in the *SalI* site of pBS-CBND are analyzed
10 by restriction enzyme analysis to determine the orientation of the insert. A plasmid with the *EcoRI* site at the 3' end of the *dhfr* expression unit is identified and designated pIFN β -1.

Plasmid pIFN β -1 is digested with *BamHI* for
15 transfection into human cells. Transfection of primary, secondary, or immortalized human cells and isolation of homologously recombinant cells expressing β -interferon may be accomplished using the methods described in U.S. Serial No. 08/243,391 and incorporated herein by reference.
20 Homologously recombinant cells may be identified by PCR screening strategy as exemplified therein and in published methods available to one skilled in the art (see, for example, Kim, H-S and Smithies, O., *Nucl. Acids Res.* 16:8887-8903 (1988)). The identification of cells
25 expressing β -interferon may also be accomplished using a variety of assays based on the structure or properties of β -interferon. For example, β -interferon may be identified by an in vitro reverse passive hemagglutination assay (Accurate Chemical Corp., Westbury, NY), stimulation of
30 superoxide anion production by mouse peritoneal macrophages (Colligan, J. E. et al. *Current Protocols in Immunology*, Wiley, New York, NY. (1994), or by using anti- β -interferon antibodies in an ELISA assay.

The isolation of cells containing amplified copies of
35 the amplifiable marker gene and the activated β -interferon

-65-

locus is performed as described in U.S. Serial No.:
07/985,586 incorporated herein by reference.

Equivalents

Those skilled in the art will recognize, or be able to
5 ascertain using not more than routine experimentation, many
equivalents to the specific embodiments of the invention
described herein. Such equivalents are intended to be
encompassed by the following claims.

-66-

CLAIMS

1. A method for controlling (e.g. altering) the expression of a structural gene in a cell comprising the steps of:
 - 5 (a) providing a DNA construct comprising a targeting sequence, a regulatory sequence and a splice donor site;
 - (b) establishing an intervening DNA sequence between the regulatory sequence and the structural gene by inserting the construct into the cell by homologous recombination at a preselected position relative to the structural gene to produce a homologously recombinant cell in which the inserted construct adopts a configuration whereby the regulatory sequence is separated from the structural gene by a preselected length of intervening DNA, the splice donor site being positioned such that cognate RNA of the intervening DNA is removed during post-transcriptional splicing of the primary transcript; and
 - 20 (c) controlling the expression of the structural gene by varying the length of the intervening DNA selected in step (b).
- 25 2. A DNA construct for use in the method of Claim 1 and capable of altering the expression of a gene encoding thrombopoietin when inserted by homologous recombination into chromosomal DNA of a cell, said construct comprising:
 - 30 (a) a targeting sequence comprising DNA which hybridizes to genomic DNA within or upstream of the thrombopoietin gene;

-67-

- (b) a regulatory sequence;
 - (c) an exon; and
 - (d) an unpaired splice-donor site.
3. The DNA construct of Claim 2 wherein the regulatory
5 sequence comprises a promoter.
4. The DNA construct of Claim 2 or Claim 3 further
comprising a selectable marker gene.
5. The DNA construct of any one of Claims 2-4 further
comprising an amplifiable marker gene.
- 10 6. The DNA construct of any one of Claims 2-5 further
comprising a second targeting sequence comprising
DNA which hybridizes to genomic DNA within or
upstream of the thrombopoietin gene.
- 15 7. The DNA construct of any one of Claims 2-6 wherein
the targeting sequence is selected from the group
consisting of SEQ ID NO: 3, SEQ ID NO: 4 or
fragment thereof or a sequence which hybridizes to
a sequence selected from the group consisting of
SEQ ID NO: 3, SEQ ID NO: 4 or fragments thereof.
- 20 8. The DNA construct of Claim 7 wherein the targeting
sequence is a fragment of SEQ ID NO: 3 and is at
least about 20 base pairs.
9. The DNA construct of Claim 7 wherein the targeting
sequence is a fragment of SEQ ID NO: 4 and is at
25 least about 20 base pairs.
10. The DNA construct of Claim 9 wherein the targeting
sequence is at least about 20 base pairs and is a

-68-

sequence between about nucleotides -1815 to -145, 14 to 245, or 374 to 570 of Figure 5 (SEQ ID NO: 4).

- 5 11. An isolated DNA molecule for use as part of the construct of any one of Claims 2-10 being of at least about 20 base pairs and selected from the group consisting of SEQ ID NO: 3, a fragment thereof, and a sequence which hybridizes to SEQ ID NO: 3.
- 10 12. An isolated DNA molecule for use as part of the construct of any one of Claims 2-10 being of at least about 20 base pairs and selected from the group consisting of a sequence between about
15 nucleotides -1815 to -145, 14 to 245, or 374 to 570 of Figure 5 (SEQ ID NO: 4), and a sequence which hybridizes to a sequence between about nucleotides -1815 to -145, 14 to 245, or 374 to 570 of Figure 5 (SEQ ID NO: 4).
- 20 13. A method of producing a homologously recombinant cell wherein the expression of the thrombopoietin gene is altered, comprising the steps of:
 - 25 (a) transfecting a cell containing the thrombopoietin gene with the DNA construct of one of Claims 2-10; and
 - (b) maintaining the transfected cell under conditions appropriate for homologous recombination.
14. A homologously recombinant cell produced by the method of Claim 13.

-69-

15. A homologously recombinant cell obtainable by the method of Claim 1 which expresses thrombopoietin comprising an exogenous regulatory region, an exogenous exon, and an exogenous unpaired splice-donor site operatively linked to an endogenous splice acceptor site of the thrombopoietin gene.
16. The homologously recombinant cell of Claim 15 wherein the exogenous regulatory region, the exogenous exon, and the exogenous unpaired splice-donor site are operatively linked to the endogenous splice acceptor site of the second or third exon of the thrombopoietin gene.
17. A method for producing thrombopoietin comprising the steps of maintaining the homologously recombinant cell of any one of Claims 14 to 16 under conditions appropriate for the production of thrombopoietin.
18. A method for producing thrombopoietin wherein the expression of the thrombopoietin gene is altered, comprising the steps of:
- (a) transfecting a cell containing the thrombopoietin gene with the DNA construct of one of Claims 2-10; and
 - (b) maintaining the transfected cell under conditions appropriate for homologous recombination; and
 - (c) maintaining the homologously recombinant cell produced in step (b) under conditions appropriate for the production of thrombopoietin.

-70-

19. A thrombopoietin produced by the method of Claim 17 or 18.
20. A pharmaceutical composition comprising the thrombopoietin of Claim 19.
- 5 21. A method of providing thrombopoietin to a mammal in need thereof comprising administering homologously recombinant cells of any one of Claims 14 to 16 in sufficient number to produce a therapeutically effective amount of thrombopoietin in the mammal.
- 10 22. A DNA construct for use in the method of Claim 1 capable of altering the expression of a gene encoding DNase I when inserted by homologous recombination into chromosomal DNA of a cell, said construct comprising:
 - 15 (a) a targeting sequence comprising DNA which hybridizes to genomic DNA within or upstream of the DNase I gene;
 - (b) a regulatory sequence;
 - (c) an exon; and
 - 20 (d) an unpaired splice-donor site.
23. The DNA construct of Claim 22 wherein the regulatory sequence comprises a promoter.
24. The DNA construct of Claim 22 or 23 further comprising a selectable marker gene.
- 25 25. The DNA construct of any one of Claims 22-24 further comprising an amplifiable marker gene.
26. The DNA construct of any one of Claims 22-25 further comprising a second targeting sequence

-71-

comprising DNA which hybridizes to genomic DNA within or upstream of the DNase I gene.

27. The DNA construct of any one of Claims 22-26 wherein the targeting sequence is selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 18 or fragments thereof or a sequence which hybridizes to a sequence selected from the group consisting of SEQ ID NO: 17, SEQ ID NO: 18 or fragments thereof.
28. The DNA construct of Claim 27 wherein the targeting sequence is a fragment of SEQ ID NO: 17 and is at least about 20 base pairs.
29. The DNA construct of Claim 27 wherein the targeting sequence is a fragment of SEQ ID NO: 18 and is at least about 20 base pairs.
30. The DNA construct of Claim 29 wherein the targeting sequence is at least about 20 base pairs and is a sequence between about nucleotides -328 to -2 of Figure 11 (SEQ ID NO: 18).
31. An isolated DNA molecule for use as part of the construct of any one of Claims 22-30 being of at least about 20 base pairs and selected from the group consisting of SEQ ID NO: 17, a fragment thereof, and a sequence which hybridizes to SEQ ID NO: 17.
32. An isolated DNA molecule for use as part of the construct of any one of Claims 22 to 30 being of at least about 20 base pairs and selected from the group consisting of a sequence between about nucleotides -328 to -2 of Figure 11 (SEQ ID NO: 18)

-72-

and a sequence which hybridizes to a sequence between about nucleotides -328 to -2 of Figure 11 (SEQ ID NO: 18).

33. A method of producing a homologously recombinant cell wherein the expression of the DNase I gene is altered, comprising the steps of:
- (a) transfecting a cell containing the DNase I gene with the DNA construct of one of Claims 22-30; and
 - (b) maintaining the transfected cell under conditions appropriate for homologous recombination.
34. A homologously recombinant cell produced by the method of Claim 33.
35. A homologously recombinant cell obtainable by the method of Claim 1 which expresses DNase I comprising an exogenous regulatory region, an exogenous exon, and an exogenous unpaired splice-donor site operatively linked to an endogenous splice acceptor site of the DNase I gene.
36. The homologously recombinant cell of Claim 35 wherein the exogenous regulatory region, the exogenous exon, and the exogenous unpaired splice-donor site are operatively linked to the endogenous splice acceptor site of the second exon of the DNase I gene.
37. A method for producing DNase I comprising the steps of maintaining the homologously recombinant cell of any one of Claims 34 to 36 under conditions appropriate for the production of DNase I.

-73-

38. A method for producing DNase I wherein the expression of the DNase I gene is altered, comprising the steps of:
- 5 (a) transfecting a cell containing the DNase I gene with the DNA construct of one of Claims 22-30; and
 - (b) maintaining the transfected cell under conditions appropriate for homologous recombination; and
 - 10 (c) maintaining the homologously recombinant cell produced in step (b) under conditions appropriate for the production of DNase I.
39. A DNase I produced by the method of Claim 37 or 38.
40. A pharmaceutical composition comprising the DNase I
15 of Claim 39.
41. A method of providing DNase I to a mammal in need thereof comprising administering homologously recombinant cells of any one of Claims 34 to 36 in sufficient number to produce a therapeutically
20 effective amount of DNase I in the mammal.
42. A DNA construct for use in the method of Claim 1 and capable of altering the expression of a gene encoding β -interferon when inserted by homologous recombination into chromosomal DNA of a cell, said
25 construct comprising:
- (a) a targeting sequence comprising DNA which hybridizes to genomic DNA within or upstream of the β -interferon gene;
 - (b) a regulatory sequence;
 - 30 (c) an exon;
 - (d) a splice-donor site;

-74-

- (e) an intron; and
- (f) a splice-acceptor site.

- 43. The DNA construct of Claim 42 wherein the regulatory sequence comprises a promoter.
- 5 44. The DNA construct of Claim 42 or 43 further comprising a selectable marker gene.
- 45. The DNA construct of any one of Claims 42-44 further comprising an amplifiable marker gene.
- 10 46. The DNA construct of any one of Claims 42-45 further comprising a second targeting sequence comprising DNA which hybridizes to genomic DNA within or upstream of the β -interferon gene.
- 15 47. The DNA construct of Claim 42 wherein the targeting sequence is selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24 or fragments thereof or a sequence which hybridizes to a sequence selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24 or fragments thereof.
- 20 48. The DNA construct of Claim 47 wherein the targeting sequence is a fragment of SEQ ID NO: 23 and is at least about 20 base pairs.
- 49. The DNA construct of Claim 47 wherein the targeting sequence is a fragment of SEQ ID NO: 24 and is at least about 20 base pairs.
- 25 50. An isolated DNA molecule for use as part of the construct of any one of Claims 42-49 being of at least about 20 base pairs and selected from the

-75-

group consisting of SEQ ID NO: 23, a fragment thereof, and a sequence which hybridizes to SEQ ID NO: 23.

51. A method of producing a homologously recombinant cell wherein the expression of the β -interferon gene is altered, comprising the steps of:
- (a) transfecting a cell containing the β -interferon gene with the DNA construct of one of Claims 42-49; and
 - (b) maintaining the transfected cell under conditions appropriate for homologous recombination.
52. A homologously recombinant cell produced by the method of Claim 51.
53. A homologously recombinant cell obtainable by the method of Claim 1 which expresses β -interferon comprising an exogenous regulatory region, an exogenous exon, an exogenous splice-donor site, and an exogenous intron and an exogenous splice acceptor site operatively linked to the β -interferon gene.
54. A method for producing β -interferon comprising the steps of maintaining the homologously recombinant cell of Claim 52 or 53 under conditions appropriate for the production of β -interferon.
55. A method for producing β -interferon wherein the expression of the β -interferon gene is altered, comprising the steps of:
- (a) transfecting a cell containing the β -interferon gene with the DNA construct of one of Claims 42-49; and

-76-

- (b) maintaining the transfected cell under conditions appropriate for homologous recombination; and
- 5 (c) maintaining the homologously recombinant cell produced in step (b) under conditions appropriate for the production of β -interferon.
56. A β -interferon produced by the method of Claim 54 or 55.
- 10 57. A pharmaceutical composition comprising the β -interferon of Claim 56.
58. A method of providing β -interferon to a mammal in need thereof comprising administering homologously recombinant cells of Claim 52 or Claim 53 in
- 15 sufficient number to produce a therapeutically effective amount of β -interferon in the mammal.
59. The DNA construct of any one of Claims 2-10, 22-30 or 42-49, isolated DNA of any one of Claims 11-12, 31-32, or 50, cell of any one of Claims 14-16, 34-
- 20 36 or 52-53, thrombopoietin of Claim 19, DNase of Claim 39, β -interferon of Claim 56, or pharmaceutical composition of Claims 20, 40 or 57 for use in therapy, for example in:
- (a) gene therapy;
- 25 (b) providing TPO to a mammal by introducing homologously recombinant cells into the mammal in a sufficient number to produce an effective amount of TPO in the mammal;
- (c) administering homologously recombinant cells
- 30 expressing DNase I to the trachea and lungs of a cystic fibrosis patient to effect in vivo

-77-

- secretion of DNase I for the relief of
respiratory distress;
- (d) implanting homologously recombinant cells
expressing β -interferon into a patient
5 suffering from multiple sclerosis to effect in
vivo secretion of β -interferon to diminish
exacerbations associated with the disease;
- (e) the delivery of TPO, β -interferon or DNase I
to a patient comprising the steps defined in
10 Claim 18, 38 or 55.
60. A graft (e.g. an autograft, allograft or xenograft)
comprising the DNA construct of any one of Claims 2-
10, 22-30 or 42-49, isolated DNA of any one of
Claims 11-12, 31-32, or 50, cell of any one of
15 Claims 14-16, 34-36 or 52-53, thrombopoietin of
Claim 19, DNase of Claim 39 or β -interferon of
Claim 56.
61. The graft of Claim 60 for use in therapy, e.g. in
the therapies recited in Claim 59 (a) to (e).
- 20 62. A pharmaceutical composition or device comprising
the DNA construct of any one of Claims 2-10, 22-30
or 42-49, isolated DNA of any one of Claims 11-12,
31-32, or 50, cell of any one of Claims 14-16, 34-
36 or 52-53, thrombopoietin of Claim 19, DNase of
25 Claim 39 or β -interferon of Claim 56, the
composition or device for example further
comprising a barrier device, a nebulizer, an
atomizer or being in a form suitable for delivery
by oral, intravenous, intramuscular, intranasal,
30 antratracheal or subcutaneous routes.

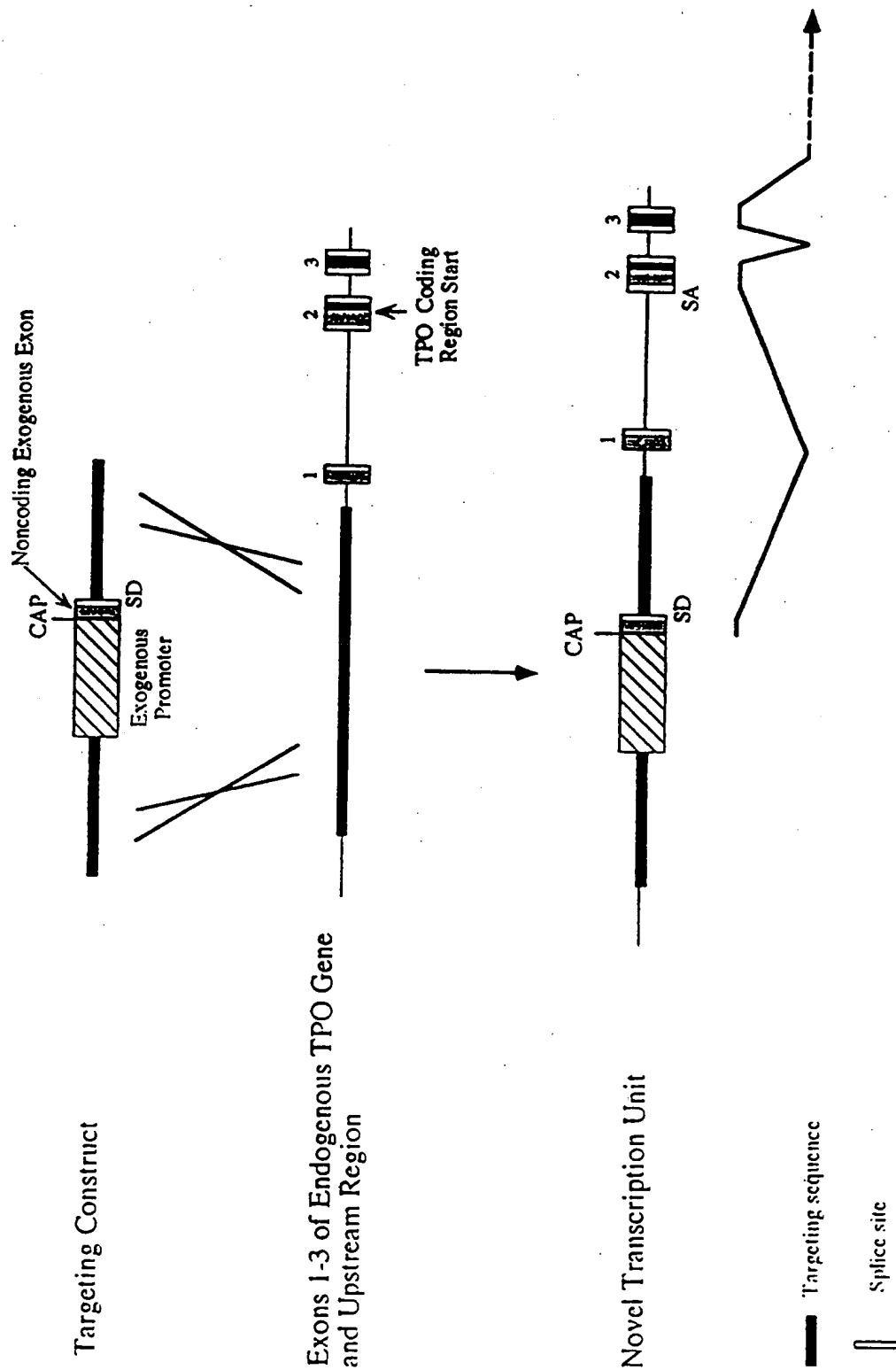


FIGURE 1

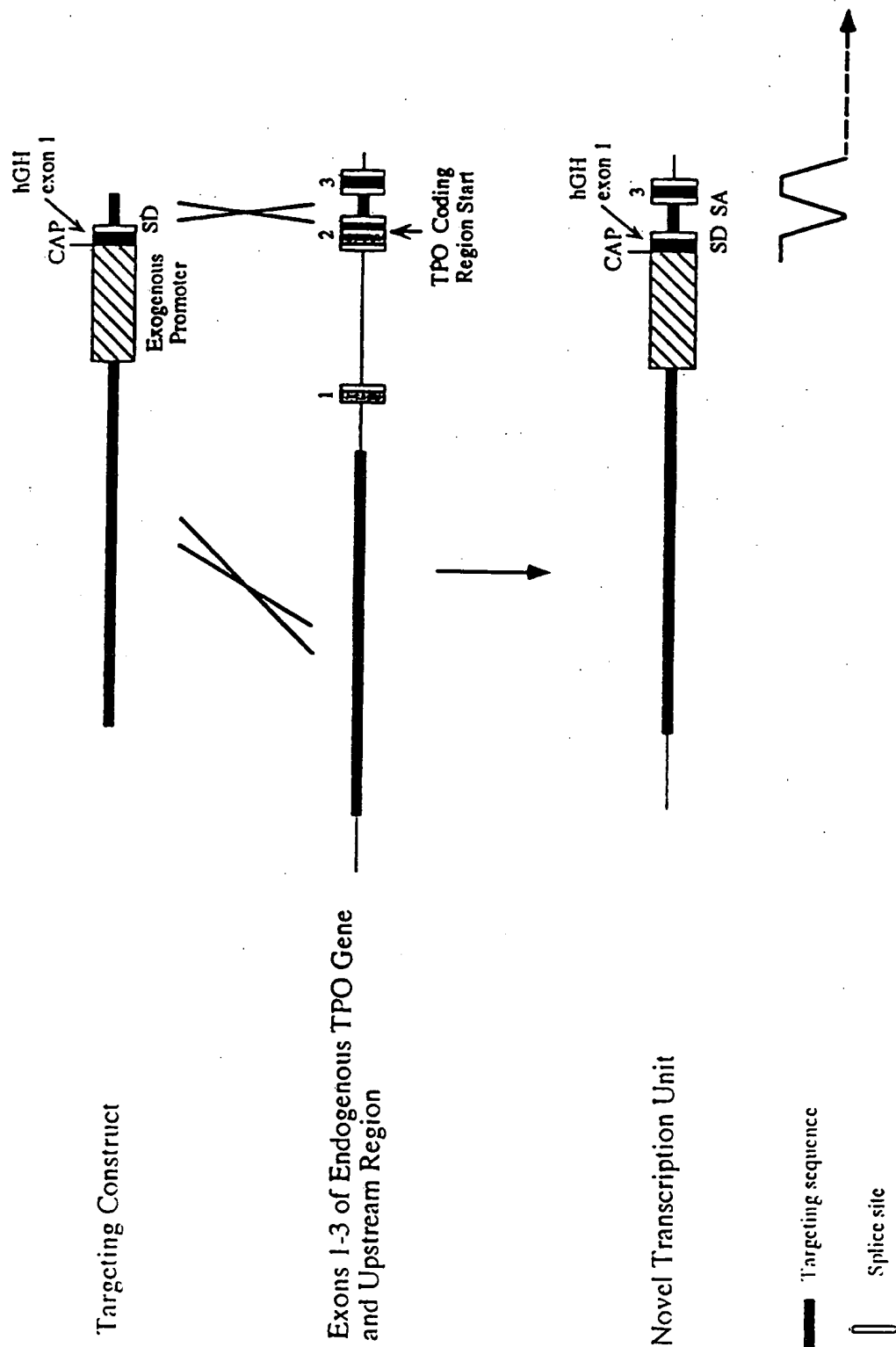


FIGURE 2

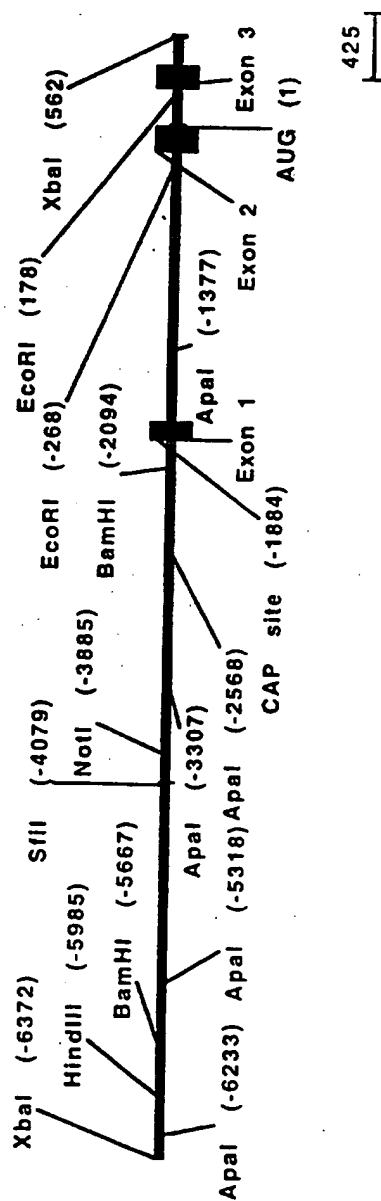


FIGURE 3

XbaI (-6372)

-6373 TCTAGAGTCAGGATGGCACTGAAGGTCTCTGGGAAGGGACGATGATGAGAGCCCCGTCAGAA
-6311 ACCCTCCCCCTTTCTCTGGGTGATAGAGAAGACTCAGAACTTCACGCCCGGGGCTCTTTGCT

ApaI (-6233)

-6249 CCCTACCTGCAGCCAGGGCCCGGTGCGATGAGAGCCCCAGACCTCCCTGAAGGGTGAGTGA
-6187 GTGTCACAAGTGCCACATGCAGCTGTTCTGCCCTAAGGAGCCGCAGAGACAACCGAGGCACT
-6125 GCCCGCCACCCCCACAGACCTGGAGCAGAGAGACAAGAAGGCCCTAGGCTCAGACACTGTG
-6063 CAGGCTAGGCCAATTAGGATGCCCAGGCAGGGCTTATGAAAAAGGAACATGGAAAGGAACCT

HindIII (-5985)

-6001 CCAGGGTGCCCTAGGAAGCTTAAGAAAGAACGCTGGAGCCAGATGCTTGGGTTCCAATCCTG
-5939 GCTGCACCACTTCCTAGCTGTGTGACCTTGAATCAAATCACATTATCCTACTGAGCCTCAGT
-5877 TCCCCCTTCTGTAAATGGGCATCATAATGTCAGTGCCCTTCTCCCACTGGGCTGTGGTGAG
-5815 GACCACGGGAGGCAATGCAGAGCATGCTCTCGGCACAGTGCCCAGACTGGGCAAGTGCTATA
-5753 AATGGCATCATCTCACCAGGCCTATCTTGGGTGRTGGGCTGCAGGGTGCTCAAACAGGAC

BamHI (-5667)

-5691 ACTGCCATTGGAGTCTGAGAAGCGGATCCTGGTAGGGCGGTCCAGCCTGGGAATGAGAGGTC
-5629 GGGTGAGGCCGGACTGAGCCAAAAGCAGCCCCCTCCCAGCTCTCCAGTTTCCCTCCSGGGCC
-5567 CGGCAGCGTGACCCCTCCTTGCTCCTTCCCCCTTCTCACCGCCTGTAGGAGATAGAGAAGCG
-5505 GAGGCTAGAGCGCCAGCAGCGAGACTCGGCTCGTGCCACCGCCTGCGACCTCGGCCCTGTCA
-5443 GCAGCGCCACGAAGTCTGGGACGGGAGGAAGATGGCCTGAGCACTGTCAAACGCCGCTTTGG
-5381 TGGCCCAGCCTCAACCACAACCCCGCTGTTGCCAGCCCCCTACCCGTGTGGCCGTCACCAC

ApaI (-5318)

-5319 GGGCCCCTCCTCAGCGCTGGCTCCCCCGGCTGCTATAACTGCGATGCTCCGGGTCCCGC
-5257 GGATACACGAAGGACAGGCCGCTCGGCTGCGCTCCGAACCTGCTGCGCTCTGCGSGGGGGG
-5195 GTAAGAACACGGGCTTCAGCTGGCCATGGGAAAGGCCAGTCCGACGCCCCATCCAAGTGGCC

FIGURE 4A

-5133 CGGGACCTAGTATCGTGGCCCTGCTCCCTCCCCGCAGCGGAGCAAGACTTACCCTGGGGGC
-5071 AGGTCTGGCAGCAGTGTCCCGGCAGCTGGCGGGCTGCCACAGGCCGGGGTTGGGCACTCT
-5009 GGTTTGATGTTCTTGACAGCTGACCTGCCAGGCCCTGGTACGGCGACCCCACTGAGGCTGC
-4947 TCCCGGAAAAGGCGGAAACCCAAGTGAGTGCAAGATGCCAACTGATGAGACCCCCCAGGC
-4885 AAGGATGTCCCGCAGAGTCAGCCAGCTCTGCCACTTACAAGCTGCGTGACCTAGACAAGCT
-4823 ACTTCATCTCTCTGGGCCTCAAGTCCCTGTCTGGAAAATGGGGATAATAATACTCTCTATC
-4761 TAGCAAGGCTGCCATGAGAGTTAGATGAGCAGGGAACGAAACGGAGTTGGCACAGAGCCTCA
-4699 CACAGAGTGGGCGATCAGTAACAGCACCTAAGAATTGGAGGGGCTGATTCCCTTCTCCAC
-4637 CAGAAAAATATCCCCAACATCTGCCGACTGGGCTCCTTCTCAGCAGCTCCGAGTCCACTCCG
-4575 ACGCCCGCGGACCCGGCCGTCCCCACCCGCCAGCCCGGGCCGGCCGGGGTGCACTCACC
-4513 GCCTCGCAGGCCACAGCACGCAGCGCATCACCCGAATGGCTCCCTAGGTCCGGGTGCCAC
-4451 GTCTCGTCCAAGGCATAGACCTTCCCGCCGAAGTGACGCTGCGGGACGGGCTTGGCTGGAG
-4389 GCGCTGCCCAGCTCGCGCCGTGTGCCGCCCGGGGGCTGCCCGGGTCCCGGTCCAGGC
-4327 ACCGCGCCCTTCTGCCCCGCCCAACCTCCGGGCGCCCGCCGCGCCGAGCCACCTGCGCCC
-4265 CGCGCCCTCTCTCCGGCTCGGCTGACTCGCCCCGAGCCGACTCCCCGCCCGCTCCCCCGG
-4203 GCGCCACCTACCTGTCTGCCGAACGGGCAGCGGCTCCTTCTCAGAACGGATGGGCAGCAC
-4141 GGGGGCTCTCGGGCCGCGGGGGCGGAGCCGAGCAGCAGCAGCCCGAGGAGCAGCAGCGG SfiI (-4079)
-4079 GCCGCGGGGCCGGGAGGGCHCGGCATGACGCGAACGGGACAGCTGGGGAGGAGGGAGGGAG
-4017 GAGGGCGCGGAGCGGGCGGAGGGAGGGAGGCGGGAGTGCGGAGGGCGGAGGGCCGGGCCG
-3955 GGGCGGTGCGGCGGAGGGGGCCGGGCGGGGCCGGGCCGGGGCAGTGCCCCGAGGGGC

FIGURE 4B

NotI (-3885)

-3893 TCGTCGGGCGGCGCAGAGTCGGCGCCGGCGGGCGGGGAGGAGCGGCGGGAGGAG
-3831 CGCGGGCGGGCGGGCGCTGACCGGGCCGTACGCGGCTCTACTGCCCCGGGCGCCGGCTCCG
-3769 GCCCGTTTTATGCCCCGCGCCGACGCCCCGCGGGGGCCTCCTCCTCAGCAAACGGGGCG
-3707 GCGGCGGCGGCTCGGCGAGGGGCGCTGAGCCCGGGGGTCCGACCCAGCAGCAGCGGCCCG
-3645 GATCGCGGTGGGGGAGGGGAGGGAGGGCTGGGACCGGGCAGGGGAGGAGGGAGGGCGGGGA
-3583 GGGGAAGGGGAGCGGGGAGGGGAGGGGAGGGAGGGACCAGGGGCGCGAAGAGGGGAGGAGA
-3521 GGCGGCCCGGAGCCCCCGCTGCTGGCGGCCACAGGGCGGCTGGACCAGGAGGTCCGTGTCCA
-3459 GCCCAGGAAGGGAGCCTCAGGCTAGGGAGGGGAGAGGCTTACCTGAGGCCTGGACCGCTCT
-3397 GTGAGCGAGGCCCGGTTCGCCCCGAAGGATAAACTTGCTTTAAAGATACACGTACAGGAAA

ApaI (-3307)

-3335 GGTCCATCAGCCGATCTCCCCCTGCCTGGGCCACAGCGCCCCCAAACCTCACCACCCTC
-3273 TCTCACTGCCTAGCCTGCCTCCCTACCTTCTCTCTGAGGTGCTCCTCWTCTTGTGTTACC
-3211 CAGRACAGGGACCTAGCCAGAAACCGGCAGCATTCCCCCTTCTGTGGAGTGACAGTATCTCC
-3149 CTCTCATTGTAACCTATCCTCAGGCGATTTCGACAGTCCCCCTCTTGCTTTCTCACCCCTTC
-3087 CTTCACCCAAGGGACCTCTGCCTCTCCAGCCCACTCCAGCCTCCTTTCTCTTGGTTCCT
-3025 GGTCAATGCCTGCCTCCCTGTCTCCTGTCTCTCCCTCCACACACCCACTATCCTCCAGC
-2963 TATCCAGCACCTCCTTCTTAATCTTGGGAGACATCTCGTCTGGCTGGACGGGAAAATTCC
-2901 AGGATCTAGGCCACACTTCTCAGCAGACATGCCCATCCTTGGGGAGGAGGAACAGGAGAGAG
-2839 CCTGAGGAAGTTCTGGGGGACAGGGGATGATGGGATCAAGGTCAGGCCAGGAAGCCCTGA
-2777 GGACAGAGACTGTGGGGAGACTTGGGACTGGGAAGAAAGCAAAGGAGCTAGAGCCAGGGCCA
-2715 AAGGAAAAGGGGGGCCAGCAGGGWGTATTTGCGGGGAGGTCCAGCAGCTGTCTTTCCTAA

FIGURE 4C

-2653 GACAGGGACACATGGGCCTGGTTATTCCCTCTGTGCACATGTGGAACGGTAGGAGATGGAAGA

Apal (-2568)

-2591 CGGAGACAGAAACAAGCAAAGGAGGGCCCTGGGCACAGAGGTCTGTGTGTGTAGCCATCTAAG

-2529 CCACTGGACCCCAGCAGACGAGCACCTAAGCTCAGGCTTAACCAGTGACGTGTGCGCACAT

-2467 ACTGTGCCCCGCACCTGACGTCCACTCAACCCGTCCAAACCCCTTCCCCATAACACCAACCC

-2405 ATAACAGGAGATTTCTCTCATGTGGGCAATATCCGTGTTCCTTCCAAAGGGGAATGAC

-2343 AAGATAGGACTCCCTAGGGGATTACAGAAAGAAAAGCAGGAAAGCAAGCATCCTGTTGGATT

-2281 TCAGCAGCAGGTATGATGTCCAGGGAAAAGAAATTTGGATAGCCAGGGAGTGAAAAGCCAC

-2219 CAATCTTAAACAAGACCTCTGTGCTTCTTCCCCAGCAACACAAATGTCCTGCCAGATTCTTC

-2157 CTGGAAAAAATCTCTGCTCCTGTCCCCCTCCAGGTCCAGGTTGCCCATGTCCAGGAAAAGAT

BamHI (-2094)

-2095 GGATCCCCCTCATCCAAATCTTCTCCGTGTGTGCTGTGGGTGGAGTGAGTRGWARCCCTGGT

-2033 CCAGGCAGGGVGCTCCAGGGAAGAGCAAGGCGTCACTTCCGGGSGGCTTCACCAGTGTCTGG

-1971 TGGCTCCCTTCTCTGATTGGGCAGAAGTGGCCCAGGCAGAGCGTATGACCTGCTGCTGTGGA

-1909 GGGGCTGTGCCCCACCGCCACATG

FIGURE 4D

-1885 TCCTCCTACCCATCTGCTCCCCAGAGGGCTGCTGCTGTGCACTTGGGTCTCTGGAGCCCTTC
-1823 TCCACCCGGTGAGTGGCCAGCAGGGTGTGGGGTTATGTGAGGGTAGAAAGGACAGCAAAGAG
-1761 AAATGGGCTCCAGCTGGGGGAGGGGAGGCAAACTGGAACCTACAGGCACTGACCTTTGTCTC
-1699 GAGAAGAGTGTAGCCTTCCCAGAAATGGGAGGAGCAGGGCAGAGCAGGGGTAGGGGGTGGGGT
-1637 GCTGKTTTCTGAGGGACTGATCACTTACTTGGTGAATACAGCACAGCCCTGGCTGGCCCT
-1575 AAGGAAAGGGGACATGAGCCCAGGGAGAAAATAAGAGAGGGAGCTGCACTTAGGGCTTAGCA
-1513 AACACAGTAGTAAGATGGACACAGCCCCAATCCCCATTCTTAGCTGGTCATTCTCTGTAGC
-1451 TTAAGGTTCTGAATCTGGTGCTGGGGAAGCTGGGCCAGGCAAGCCAGGGCGCAAGGAGAGGG
Apal (-1377)
-1389 TAATGGGAGGAGGGCCCACTCATGTTGACAGACCTACAGGAAATCCCAATATTGAATCAGGT
-1327 GCAAGCCTCTTTGCACAACCTTGTGAAAGGAGGAGGAAGCCATGTGGGGGGTCTGTGAAGGA
-1265 ACCGGAAGGGTTCTGCCAAGGGGGCAGGGAGGCAGGTGTGAGCTATGAGACAGATATGTTA
-1203 GTGGGCGCCTAAGACAAGGTAAGCCCCCTAAGGTGGGCATCAGCCAGCAGGTGCCCGTTCTCTG
-1141 GGCAGCTGGTTTCAGGAAGGAAGTCCCAGAACTGTTAGCCCATCTCTTGGCCTCAGATAATG
-1079 GAGTATTTTCAGGACTTGGAGTCCAGAGAAAAGCTCCAGTGGCTTTATGTGTGGGGGTAGATA
-1017 GGGAAAGAATAGAGGTTAATTTCTCCCATACCGCCTTTTAACTCTGACCTCTAGTGGTCCCA
-955 GTTACAGCTTTGTGCAGTTCCCTCCCCAGCCCCACTCCCCACCGCAGAAAGTTACCCCTCAA
-893 CATATTGCGCCCGTTTGCCAGTTCTCTACCCAGGCCCTGCATCCCATTTTCCACTCTCTTCT
-831 CCAGGCTGAAGCCACAATACTTTCTCTCTATCCCCATCCCAGATTTTCTCTGACCTAACA
-769 ACCAAGGTTGCTCAGAATTTAAGGCTAATTAAGATATGTGTGTATACATATCATGTCTCTGCT
-707 GCTCTCAGCAGGGGTAGGTGGCACCAAATCCATGTCCGATTCACTGAGGAGTCTTGACAAAA

FIGURE 5A

9/30

-645 AGGAGACACCATATGCTTTCTTGCCTTTCTTTCTTTCTTTCTTTCTTTCTTTTTTTTTTTTGAG
-582 ACGGAGTTTCACTCTTATTGCCCAGGCTGGAGTGCAATGGTGCGATCTCGGCTCACCCACAACC
-519 TCCGCCTCCCAGGTACAAGCGATTCTCCTGTCTCAGCCTCCCCAAGTAGCTTGGATTACAGGCA
-456 TGAACCACCACACCCTGCTAGTTTTTTTTGTATTTCGTAGAGCCGGGGTTTCACCATGTTAGTG
-393 AGGCTGGTGGCGAACTCCTGACCTCAGGTGATCCACCCGCCCTTGGACTCCCAAAGTGCTGGGA
-330 TTACAGGCATGAGCCACTGCACCCGGCACACCATATGCTTTTCATCACAAGRAAATGTGAGAGA EcoRI (-268)
-267 ATT CAGGGCTTTGGCAGTTCCAGGCTGGTCAGCATCTCAAGCCCTCCCCAGCATCTGTT CACC
-204 CTGCCAGGCAGTCTCTTCTCTAGAAACTTGGTTAAATGTTCACTCTTCTTGCTACTTT CAGGAT
-141 AGATTCTTTACCCCTTGGTCCGCCTTGGCCCCACCTTACTCTGCCCAGAAAGTGCAAGAGCCTAA
-78 GCCGCCTCCATGGCCCCAGGAAGGATTCAGGGGAGAGGCCCCCAAACAGGGAGCCACGCCAGCC

AUG (1)

-15 AGACACCCCGGCCAGA ATG GAG CTG ACT G GTGAGAACACACCTGAGGGGCTAGGGCC
43 ATATGGAACATGACAGAAGGGGAGAGAGAAAGGAGACACGCTGCAGGGGGCAGGAAGCTGGG
106 GGAACCCATTCTCCCAAAAATAAGGGGTCTGAGGGGTGGATTCCCTGGGTTTCAGGTCTGGGT

EcoRI (178)

169 CCTGAATGGGAATTCTTGAATACCAGCTGACAATGATTTCTCTCTCATCTTTCAACCTCACC
232 TCTCTCATCTAAGAA TIG CTC CTC GTG GTC ATG CTT CTC CTA ACT GCA
281 AGG CTA ACC CTG TCC AGC CCG GCT CCT OCT GCT TGT GAC CTC CGA GTC
329 CTC AGT AAA CTG CTT GGT GAC TCC CAT GTC CTT CAC AGC AGA CTG GTG
377 AGAACTCCCAACATTATCCCTTTTATCCGCGTAAC TG GTAAGACACCCATACTCCCAGGAAGA
440 CACCATCACTTCTCTAACTCCTTGACCCAATGACTATTTCTTCCCATATTGTCCCCACCTACT

XbaI (562)

503 GATCACA CTCTCTGACAAGGATTATCTTTCACAATACAGCCCGCATTTAAAAGCTCTCGTCTA
566 GAAC T

FIGURE 5B

10/30

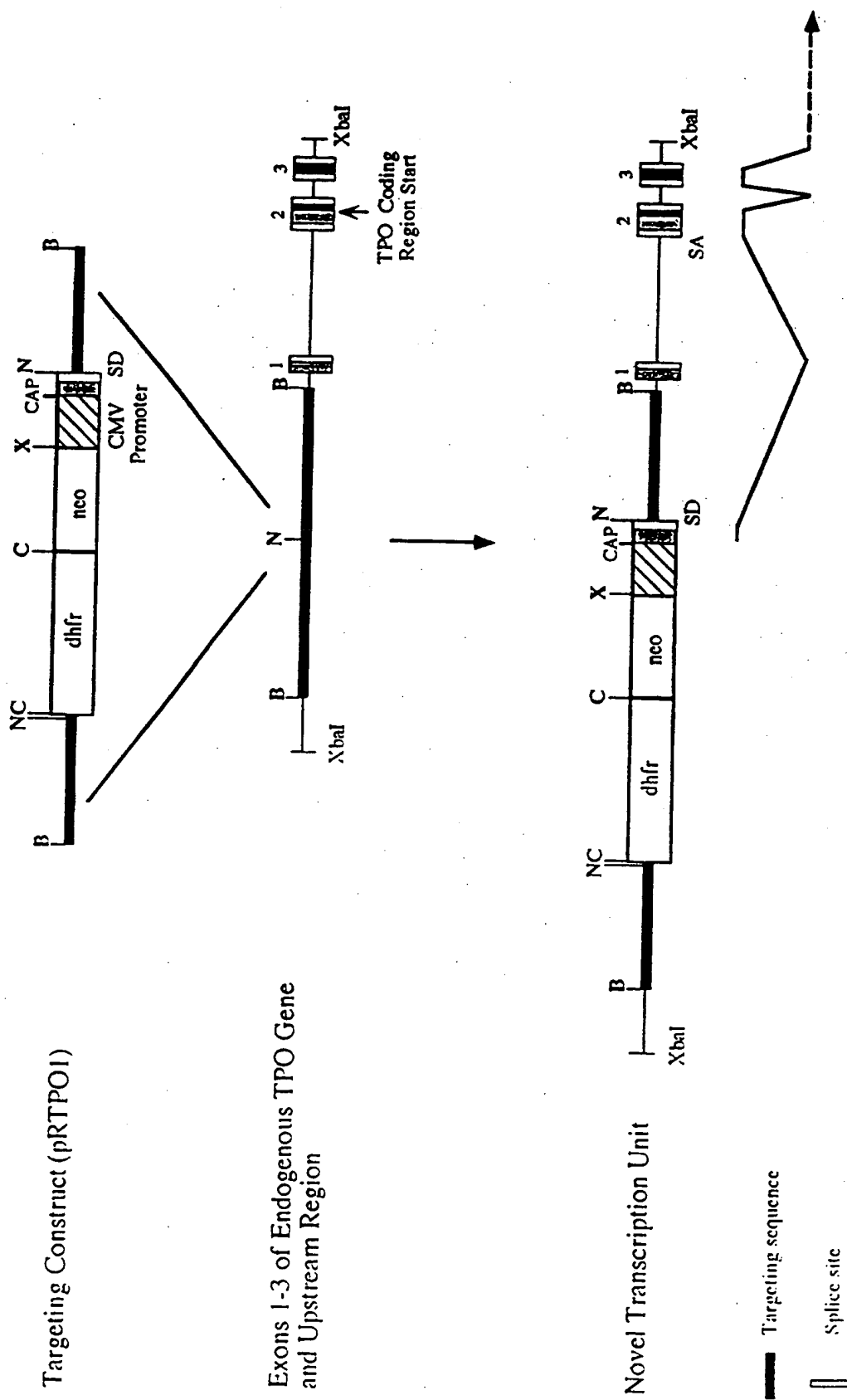
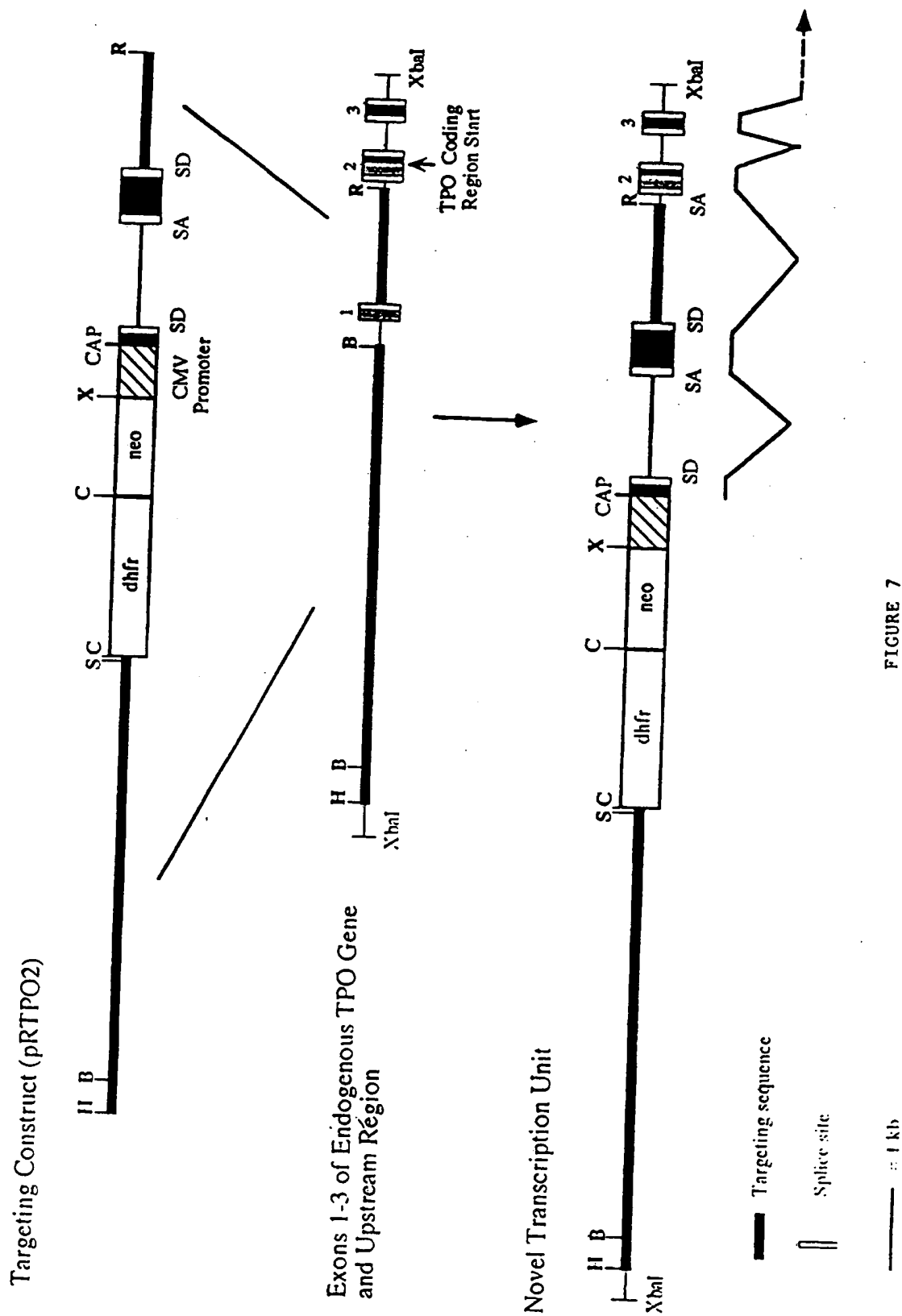


FIGURE 6

11/30



12/30

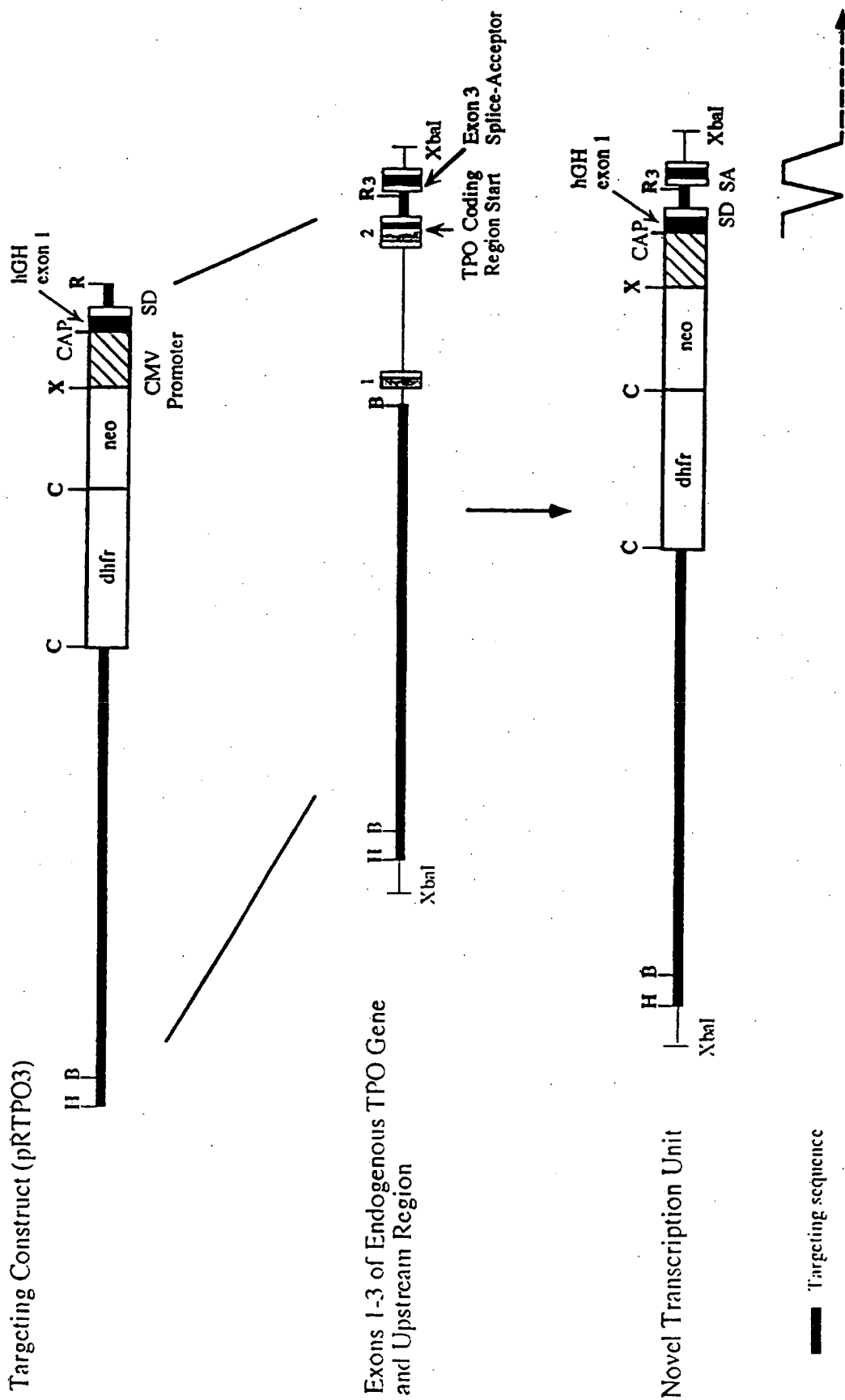


FIGURE 8

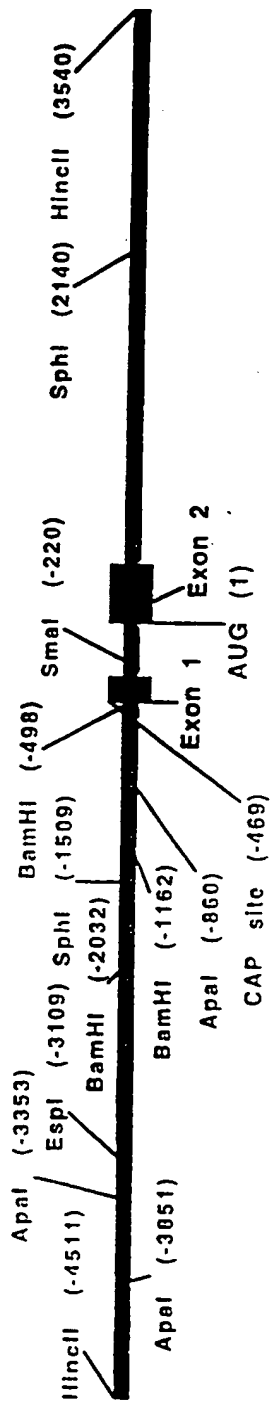


FIGURE 9

HincII (-4511)

-4512 GTCAACCTTCACAGTAATTGCTTGTTCACTGACTGCCACAACCCAGCCTGGCAGAGAGAGGGAA
-4448 GATACCTTATAAAGCAAGGTAACGTTAATGTTGAGACCATGAATGGCCTTGAGCAGAGCAGAGT
-4384 ATCATTCGCTTCCTTCAAATTCAGAAGGATCTGATGGTGCTCTGTGAGTTCATGGGGGTGCCTC
-4320 CGTCAGGTTGAAACCACAGCTGTCGCTCCTTCGGCTTTCCCTCTTGATCAGTAGAAGGGTACCC
-4256 TCCCTGGCCTGCACGTCGCTGGGTCAACAACACTGGCTGTCGTTGCACAAAGCCACGGCCACC
-4192 AGCGTTCCCTTTGAGGCCATTTGTTTCCAGCCATGGTGCCTATAGGATTTTTCCTTTATCCGTGA
-4128 ATTTTCAGCCAAATCAGAGCATGTGACCTGGCTTAGATGTCAATATAATGTTGTTATGTGCTCT
-4064 TTTCCCTTCCTGTGTCTGTGACAGGTTTAATTTAACTGAGAAGGCTGCAGATCCTCGGGGGTT
-4000 GGTGTAAAAACACCTCATCTGATCTGAGAAGGCGGTGAGCTTTTCTCCTGGTTGCCGTTGGCT
-3936 GCCAGCACCCATTCTCTGTGGATGTGAAAATCCCAGAAGGGCTGGGCTTCTTCTTGGCATTCC

ApaI (-3851)

-3872 CCAGGCCTATCTCCAGAGTGGGGCCCAGCATGGGAGGATTGTACCCCACTCACTCCCTGATGT
-3808 GGGGCTTGACCTACAGCTCGACAGCACCCATGGAATGTGGGCAGAAGCGACAGCAGCCAACGT
-3744 CCGCCTTGCCCTTAGGGCGGCACGTGTTCTGCTGTGCCCCTGGGAGCCTCCACCTTCCCACTG
-3680 TGGGAAGAGGGTGCCAGGGAGCTGCAGTCTCTCCAGCCCAGCCCCAGGACGAGGCCAGGCAG
-3616 CAGAGCCACCCAGCAGACCTGGCAGTGTGAGAGAAATGCATGTGTATACACTGAGTTTGCAGG
-3552 TGGCTGTTACATGGCAGCATTGACTGACACAGACAGAAAAGAGATCCACGAGGGAGAAGTGAGA
-3488 GTGCTGGAGACTCCAACAAGCCACAGGCTGCAGGGGCAGGATGGCTTCTTAGAAGGTGAATGAT
-3424 TGTCTGGGAATCTATCAGAGGAAGACATAGAGGCTCCAGACGGTTGAAGGCCCAACAGTGATC

ApaI (-3353)

-3360 CCAGACGGGCCCCATGTCAGACCAGGCTCCTCCAGGGCTGTGCTGCCCTCACCAAGGCCCGTC
-3296 CTGAGGGCAGCCACACAGCAGGCAGCACTCGCCATTTGTACAAGCGAGGCCCAAGTTCCAGCCT

FIGURE 10A

-3232 TCCTTCTGGCAGGTAGAGGAAGCAGGGGCACTATGCCTGGGAGTTCCTGAAAGCAGATGGGGCA
-3168 GCATTTGGTCAAGAGCCAGGAGGGGATGACAGACCAGAGGGGAACCCCTCGTCCACGTGCTGAG
Espl (-3109)
-3104 CACACGTAGGGGGTTGGGCACTTGCTCTGTGAGCTATAATTGGTGTCCCTGTGCCCCGCCGGAA
-3040 GCTGCACCAGGCAGTTTCTTGGTGGAGGACAGTGGCCGCCCTCTAGCTTTACTCCCTTCCCGT
-2976 GATGGGTGCTGTGAGATGTGTGTCCAGGAAAGGCAACACCAAAGGCAGAGGACTAGTCCCTA
-2912 CACCGAATACTCCGGTGGCCTTGCTTGGGGGCTGGGTTTGTACGTGCTGGAGGCTGTCTTAGAC
-2848 TTAGAGATTAAAAACAGGAAGAACCATTGCTGAAACCTTTGGAAAAGCCTGCAATGGCCTCTG
-2784 GCAGCCTGAGGAGTGGTGGTGTTCATCTGGTAGACGCCGTCTCAATAGGAGGGACAGATGAG
-2720 TGCACCAGTGCTGCCAGCCAGAGGCGTCTGTGGCGTGTCTTTATGGAATGGGGTGCCAGTCTT
-2656 GTGGAGGGTGGTTTACCTTCTGTCTTAGTCCCCACTGGGCGTGCCTTCTGCTTCATGCCAGC
-2592 TGGCCAGACCGAGCACTTTCCTGACTTTCGACCTTGGCCCCCTGCTGACTCTTGCCGTGAGGCC
-2528 TCCTGCAGACCCCATTTGTATTCACTTCCTGCAGTCTCATACCTGAATCCCGCCTGGACTTCT
-2464 GCCAACCGTCCAGGCCCTCTCCAGGGGGACCAAGATGCTACGTGCAGGGCTGTCTTGGA
-2400 GGGCCAGCACAGCCCCCTTCCAAGTGGGCAAGACCCAGGGGTGGCTCAAAAGATAGCTGTGCCCT
-2336 AGCCCTGGAACCTCTGAATGTTGATTTTTGTAGCAAAAAGGACTTGCAGATGTGAGTAAAGGC
-2272 TGTTGAGATAAGGACATCCTCCCTGCTCTCTGGGAGGACCCAAATGCAGGTGCACAGATCTTA
-2208 AGAAGAAGAGGCAGAGACTGGGGTGATGCAGCCACAATAAGGAAAGCCAAGGATTGCTGGCAG
-2144 CCTGCAGAACTGGAGGGCAAGGAGCATCCCCCAACCGCCCGAGCCTCCAGGAGGCGCAAGGT
-2080 CCTACTGACTCCCTGACTTCAGACGTCCAGTCTCCGGAATTTGAGAGGATCCATTTCTGTTAT
BamHI (-2032)
-2016 TTTAAGCAACCAAACCTTGTTGGTAGTTTCACCACTCTCAGGAAATGAATACGAATGGAAAGTCAA

FIGURE 10B

-1952 AGATTCCAAGAAATGAGTGGCGGGGTGCGGTGGCTCACACTTGTAATCCCAGCATTGCGGGAA
-1888 GATTGCTTGGGCTCAGGACTTGGAGACCTTGTGTCTGTGAGAACTTAAAAATAGGCTGGGTG
-1824 CGATCGTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCAGGCGGATCACAAGGTCACGA
-1760 GTTTGAGACCAGTGTGACCAACATGGTGAAACCTGTCTCTACTAAAAATACAAAAATTAGCCG
-1696 GGTGTGGTGGTGGTGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGAAGAATTGCTTGAA
-1632 CCCAGGAAGCAGAGGTTGCAGTGAGCCGAGATAGTATTACTGCACTCCAGGCTGGGCAGCAGAG
-1568 CAAGATTCGCCTCAAAAAAAAAAAAAAAAAAAAAAAAAAACTGAGCATGGTAGCATGC SphI (-1509)
-1504 ACCTGTGGTCTCGTACGCCGAGGATTGCTGAAGCCAGGAGTTCAAGACCAGTCTGGACAAA
-1440 AGAGCAAGACCCCATCTCTACCAAAAAATTTAAAAATTAGCCAGGCATGGTGCCGTACCCATA
-1376 GTCTTAGCTACTCAGGAGGCTGAGGAGGGAGGATTATCTGAGCCTGGCGGTTGAGGCTATAATG
-1312 AGCCATGATTTGGCCACTGCACTCCAGCCTTGGCAACACAGTGTGAGACCTGTCTCAAAAACA
-1248 ATAAAAACCCAAACAAAAGAACCAAGAAATTACTGGACCTGAGCCTGGCCTTTAGCTGCTGCC
-1184 CTGCCCTKTGACTGGTCACTCGGATCCCTGGGCCTAAACACACAGCCTATTGTCTACCTCAAGA BamHI (-1162)
-1120 AGGCTCCCCACTGCTTGGCTGGCAATTGGGGTGGCTTTGCAGGCCCCACCTGTCTGGCCCCAC
-1056 GGCCTGGTGTGCTGAGGCCCCCACCCTGCTTGTTCGAGCTCCCCAGCCTCCTGCAGAGTTGC
-992 CTGCACCTGATGGCGATGAATCAGGAAGGCAGGCGTGTCTGGGCCACAGAGCAGTCATGCTGT
-928 CAGCCACCAGGGGGCTCCATTTGCAACTTTGGATGTGGCTTTGGCCTCTTTGTCCAAAGTGACC
-864 TTGGGGCCCCCAGACAAGAGACAGGGAGACTGGAGCCCAGCCCCACCTCCCCGCACATACCTGG ApaI (-860)
-800 CCCATCCCTGCCCTATCCTGGAAGATGGGGGCCACCACACGTRCAAGGGACACGGGATAGGAAC
-736 CTTTGGCCTTGTATCAGACATTTTAAAACTAAGTGCAAACGTGATTATCAGGTGCAGTTTATA

FIGURE 10C

17/30

-672 CAGCAGCAAGAAACCTGTGCTTACAGAAAGAAACACGTGCTAGCAACCCACCTATGCGGAAAGCC

-607 ACACAGAGCCATTGTTTTCTGCACTCTCAGGTGACGGCTCACATTTGCCCCAGGGAAGGTCACAG

-542 CTGCCTGAAC TTTTAAAACTCCCAGACAGCACTGCCTGTGCAGGATCCGGAGCCCAGCAGCACT
BamHI (-498)

-477 GCCAGGG

FIGURE 10D

CAP site (-469)

-470 CCTTGAAGTCTTCTTCAGAGACCTTTCTTCATAGACTACTTTTTTCTTTAAGCAGCAAA
 -408 AGGAGAAAATTGTTCATCAAAGGATATTCAGATTCTTGACAGCATTCTCGTCATCTCTGAGG
 -346 ACATCACCATCATCTCAGGTGAGCAACCAGGTGGAGTGCCCTCTGGGTGACTGGCCGGTTTGGGA
 -284 GCAGGGAGGGAGGCTTAGAGTCTCATCTCCAGCAGCGAGTGAGGCGGAGGCTCCAGCGTCC

SmaI (-220)

-222 TCCCCGGCGGGTTTTCTGGTGGATGGAGGAGTGACTCGGGGTCTCTACGTGGTGCCAGCTG
 -160 TTTGGCTTTCTGGACGTTGTAGGAAAGGGTTTCCCCCGCCTGCGTCCCCCTGACCTTGAGCT
 -98 CCACCAGCCCCCTGCCAGCTGGGCTCCAGAAGGCTGGAGTGCTGTGGCAGGGATGACGTCTCA

AUG (1)

-36 CTTCTGTATGTCTCTGTGCCCTGTGCTCTCCAGG ATG AGG GGC ATG AAG CTG
 19 CTG GGG GGG CTG CTG GCA CTG GGG GCC CTA CTG CAG GGG GCC GTG
 64 TCC CTG AAG ATC GCA GCC TTC AAC ATC CAG ACA TTT GGG GAG ACC
 109 AAG ATG TCC AAT GCC ACC CTC GTC AGC TAC ATT GTG CAG ATC CTG
 154 AGC GGC TAT GAC ATC GCC CTG GTC CAG GAG GTC AGA GAC AGC CAC
 199 CTG ACT GCC GTG GGG AAG CTG CTG GAC AAC CTC AAT CAG GAT GCA
 244 CCA GAC ACC TAT CAC TAC GTG GTC AGT GAG CCA CTG GGA CGG AAC
 289 AGC TAT AAG GAG GGC TAC CTG TTC GTG TAC AGG CCT GAC CAG GTG
 334 TCT GCG G

FIGURE 11

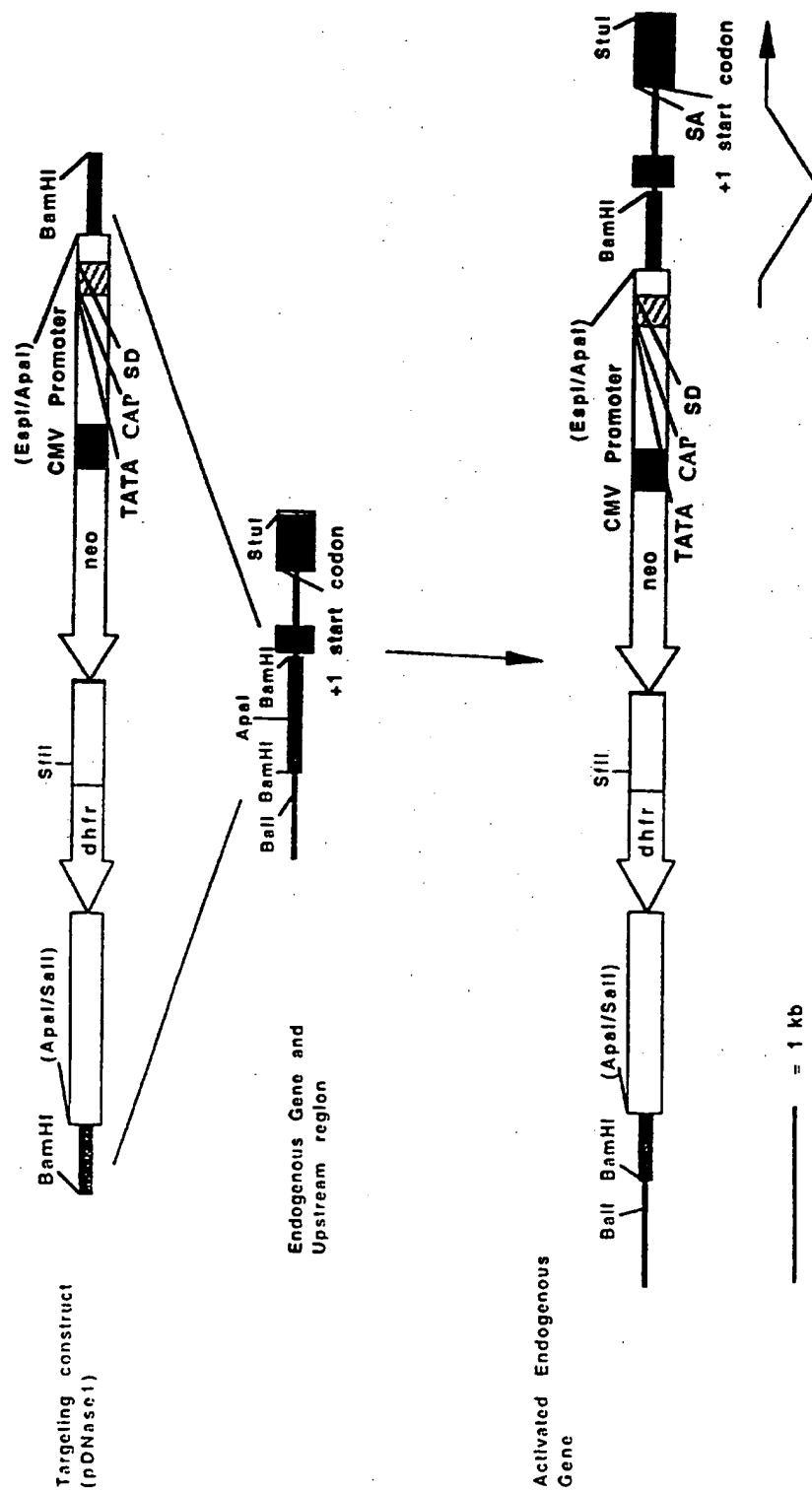


FIGURE 12

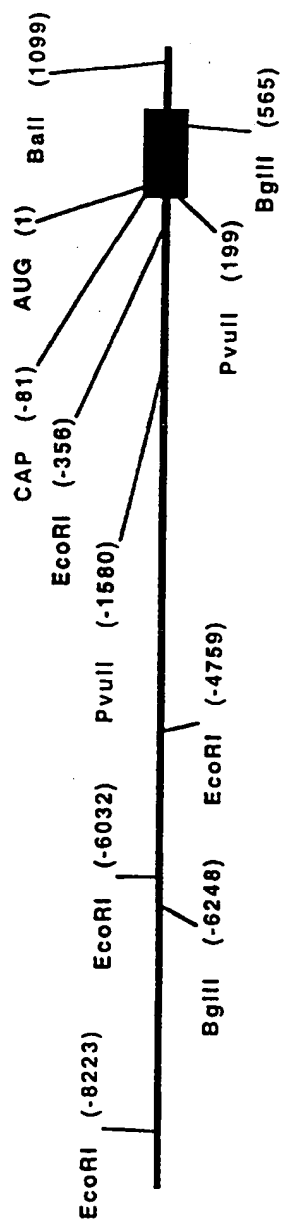


FIGURE 13

-8711 AGCTTCTGCTTTAGGAAAGTAGAAAAATAAGAGCAAATTAAATCCAAGGTAAGTAAAAAAAAAAAA
-8646 AAAAAAAAAAAGAAATAAAAATTAGAGCAGAAATCAATAAAATTGAAGACAGTAAATCAATAAA
-8581 GAAATCAACATAAAAAGTCTGGTTCTTGAAAAGATATATAAAATTGATAAGCATCTACCTAGGA
-8516 TAATTAAGGAAAAAAGACAGAGGACACAGATTACTAATATCAAACATAAAAGCGGGAACATCACT
-8451 GCAAATTTTATAGGCATTGAAAGCGTAATAAAGAATACTATAAACTATTCTATAACTACAAATT
-8386 TGATAAGTAAATAGAATGAACCAATTCCTTGAAAGACATAATCTGAAAAATGTAAAAAGAAGAAA
-8321 TAAACAATCTGAATAGCCTATATCTATTAAATAAATTGAATCAGTAATTAAACCTCTCAAAAC
EcoRI (-8223)
-8256 AGGAAGCACAATGCCCAGATGGGTTCACTAGTGAATTCATCAAATATTTAAAGAAAAAAAAAATT
-8191 GTATCAACTTTCTACAATCTCTTTTCAAGACAGAAGCAGAGGGAATACTTCCTAAATCATTCAA
-8126 CTAGGCCAGCATTACCTTAATACCGGAAGTAGAAAATGACATTACAAGAAAAGAAAACAACAGAC
-8061 CAATATCTCTCATGAACAAAGATACAAACATTTTCAACAAAATATTAGCAAAAAGAATCCAAGAA
-7996 TGTATCAAAAAATATACACCACAACCAAGTAGAATTTATTCCAGATATGTAAGGGTGGTTCAACG
-7931 TTTGAAAATCAATTAAACGTAATTTGTCCCATCAACAGGTTAAAGAAGAAAATCACATGGTCATAT
-7866 TGATAGACACAGAAAAAGCATTGACAAAATTTAACACCCATTTCATGATGCAATCTCTCAGTAAA
-7801 CTAGGAATAGAGGAAAACCTTCCTCAGCTTGAATGTACCTTCCTCTCAATTTTGCTATGAACCTGA
-7736 AACTCCTCTTAAAAAATAAAGTTTTTCATTTAAAAAGAAAACAAAAACATGGAGGAGCGTTGAT
-7671 GTATCTCATTTTAGACCAATCAGCTATGGATAGTTAGGCGACAGCACAGATAGCTGCTGTACTTC
-7606 TGTCTCTGGCAATGTTCCAGACTACATTTAAAAAATTTTAAATTATAGACTTGTAATGTTTC
-7541 AAGAAAAATATGAAAATGCTTTGCCGTGTTAATGCTACTCTTTTAAAAAAAACATAAGTTCAA
-7476 ACTTTATTTATATTTTATTAGTTTCTAGCTACTGTTCTTTTCTGTTCTGGGATCTCATTTCAGA

FIGURE 14A

-7411 ATGCCACATTACATATAATTCTCATGTCTCCTTGGGTTCCTCTTAGTTTTGACAGTTCCTCAGAC
-7346 TTTTCTTATTTTGTGATGACCTTGACAGTTTGTAGGAGTACTGGTTAGATATAGGGTAATGGTTTT
-7281 TAAAGTATATTTGTCATGATTATATACTGGGTAAGGGTTTGGGAGGAAGCCATGGGTAAGTACTGT
-7216 TCTCATCAGATCATATCAAGTTATATACCATCAATATTGCCACAGATGTTACTTAGCCTTTTAAT
-7151 ATTTCTCTAATTTAGTGTATATGCAATGATAGTTCTCTGATTCTGAGATTGAGTTTCTCATGTG
-7086 TAATGATTATTTAGAGTTTCTCTTTTCATCTGTTCAAATTTTGTCTAGTTTTATTTTTACTGATT
-7021 TGTAAGACTTCTTTTTATAATCTGCATATTACAATTTCTTTACTGGGGGTGTGCAAATATTTT
-6956 CTGTCATTCTATGGCCTGACTTTTCTTAATGGTTTTTTAAATTTTAAAAATAAGTCTTAATATTCA
-6891 TGCAATCTAATTAACAATCTTTTCTTTGTGGTTAGGACTTTGAGTCATAAGAAATTTTCTCTAC
-6826 ACTGAAGTCATGATGGCATGCTTCTATATTATTTTCTAAAAGATTTAAAGTTTTCCTTCTCCAT
-6761 TTAGACTTATAATTCAGTGAATTTTTTGTGTGTATGGTATGACATATGGGTTCCTTTTATTT
-6696 TTTACATATAAATATATTTCCCTGTTTTTCTAAAAAAGAAAAAGATCATCATTTTCCCATGTAA
-6631 AATGCCATATTTTTTTCATAGGTCACTTACATATATCAATGGGTCTGTTTCTGAGCTCTACTCTA
-6566 TTTATCAGCCTCACTGTCTATCCCCACACATCTCATGCTTTGCTCTAAATCTTGATATTTAGTGG
-6501 AACATTCTTTCCCATTTTGTCTTACAAGAATATTTTGTATTGTCTTTTGGGCTTCTATATACA
-6436 TTTTAGAATGAGGTGGCAAGTTAACAAACAGCTTTTTTGGGGTGAACATATTGACTACAAATTT
-6371 ATGTGAAAAGAAAGTATACCTTCACAATATTAAGTCTTTTAGTTCATGAATATAGTATGTCTCTC
-6306 CGTTTCTGCATTAACTTAGACATTCATTAATTTCTCTCACAATTTATAAGTTTATTTAGATCTTC
-6241 ATTCATTTAAATCTTCACTAACCTCTCATTTACAATTTGTAAGTTTCTGGGTAACAGTCTTGCA
-6176 CTTCTTTGCCTAGATTTATTTCCAAGTAGATTATTTTCATACATCGTCTATGGTGTCATTTTTAA

BglII (-6248)

FIGURE 14B

-6111 AATGTAATTTTTCACCTTTTATTGCTAAAGAGAGATGACTGATTGTTAATATTGATCTTGTGCC

EcoRI (-6032)

-6046 TGGCGACCTTGCTGAATTCCTAATCGTTTATCTATAAATTCCTTTGTATTTTGAATGTAAACAATT

-5981 AGATCATCTGCATATAATTTTTAAATCTGATAAGTCAACAAGAGATTGAAACAGGCTCTTCACA

-5916 AAGAAAATATCCAAATGGTCAATAACATATGAAAAGATGCTGAAACTTGTTAATAATCAGAGAG

-5851 ATGCAAATTAACTATAATGAAGTATTATTGTACAACAATAGAATGACTGAAATTAAAAAGACTG

-5786 ACAATATCAAAGTTGGCAAGAGTCTGATACAACCTGGAACCTCTCAAACACTGTTAGTAAGAATGT

-5721 AAATTGGTACAAACATTTGGGAAGTCATTACAATATTATCTGCTAAATCTGAACATATACATATT

-5656 CTATGAGCCAGTTACTTCATTCTAGGCATATACCCAAAAGAAGTATGTACTATTGTGCAGTAAAA

-5591 AATACAGACAAGGAATTCATAGGAGCATTAAATTATCATGGCAAATATTTTAAAAAATTATTAGT

-5526 AGTAGAAGGGATAAAACATTGTGGTATACTTCTAAATAGGGTAAAACACATTAATGTAAATTAAT

-5461 AAATATACACACAAGATAGACGAATTTCCGACACATTCGTTGAGGGTAAGAAGACCATTTATA

-5396 CAAAGCTCAAAAACAGACAGAATCTAGAGTGTTAAAAGACTGCATGGTAGTGACTTTGGGAGAAG

-5331 AAAGTAGTGACGAGAGAGAGGAGAGAGAATAATGATTGCGAGGTGCTATAGTCTGAAGGTTTGTG

-5266 TCCCCCAAATTTACATGTTAAAACCTAATCCCCAATGCAATCATTTTAAGAAGTGGGTCTTTA

-5201 GTGGATAATTAGGTAATGGAACAAGAGCCCTAACAAATGGGATTGGTGCTTATAAAAAGAAGCCT

-5136 GAGCCTGAGGGACCTTGTTTCCCGCTTCTACCATATGAGAATGCAATGAGAAGGCACAAAGCAAA

-5071 GAGCAAGCCCTCATCAGACACTGAATCTGCTAGGGCCTTAGTCTTGGCTTTTCCAACCTCCAGAA

-5006 CTATAAAAAGAAATGCTTGTTGTTTAAAAGGCATTCAGTCTATCGGTGTTTGTGTTAGACGAGCCC

-4941 CAAGAGACTTAAGAGGGAACAAGAGGGCGATTTCTGTTGTGTTGATAATGTTTAGTTTGTGGTTA

-4876 CAAAGAGTGCAGACGTTTTTATTTTATAACAATTCATTGAGCTATATCTTAAGATGTATGCGTAA

FIGURE 14C

EcoRI (-4759)

-4811 TTTTCTATGTATATTATTGTTTATAAACTTTTCTTAAAAGAGGAAATGGGAATTCTCCCTTTT

-4746 ATGTATTAATCTCTTATGAAAGAGTTTGTGGCTTCCCAAGATATTTCTGAAAGATTGCTTTTGG

-4681 CTTCAATTTATGTTCTGCCACTGCTTATGCACCTCTCAATAACTCTTCATCTTGTATAATTTATCA

-4616 TTCTTTGATAGGGACCCTCTTCCTTGAAAAATAATTGAAGATATAAGGAGGAGGAAGAGAAGACA

-4551 ACTAAATGTTTATTTCTAGATACATAGTAGTCTGCATAGATAATTATATTCAAAGAGGAGGACA

-4486 AATTGGCTCCTATCTCTGAAATTTATAGAAAAGCATTTCACATTAAAGTGATTTCAAATGACTA

-4421 GAAATGTCATTCAAGTTTACTTTCTAAATGTCACTCTGTCTCTCCAAACCTCATTAAACCACAAG

-4356 GAACTGGTGCAGGGACTGGAAGTAGTTTCTCATACAACGGAAAGTTAACGAGGGGAGGAAAGGA

-4291 TGTGTGCAAAAATAACGTCCACAGAAGGGACAAATAACAAAGGGAAGATGACAGGAAAGGGTTC

-4226 GGGCACTAACCCCTTACAATGCAGATACACACTGGGCTGGTCTAAGAAATAGGGTTCCCTGGTAGA

-4161 CAGAAGGTTAAATAAATTTTCTGGTTATTCTGATACAACCTCTAATAAAAGAAGAGAAATGAAGC

-4096 TAAAACTTAAATGATGTATTTAAAAGGAAGAAATTTTAACCCATTTCATAGGTGAGCTTCTGCCA

-4031 AGATTACTACTAATCCTCAGGAGAAGGGGTAGAGGAGAACTCCATAAAGGCAACTGGAAGTGGA

-3966 GTATTAGGAAGCACCTCAAGAACACAATAGCAGGAAGTAGCTAGAGAACAAAGAGAAGAAAACCA

-3901 GAAAAAAAAAATCCCTTTTTTATTTTCTGTTTCCATTCTCTTGGCTCCATTTCACAGCTATGGC

-3836 CTTTATTTTCACCCTCCACAGCCATGAGAGCCTCTGGGCAGGAGTTCTCCTCGCCTCTCCCTGTT

-3771 CCAATCACCTCTAACATTTCTGCCTATTGTTCTGCCCAGGGAAAAAACTCCAGTCTCTTCTCTGT

-3706 CAAAGACCTCTTGAATTAAGTCCAAATGCTACACTCTGGCATTCAAGACTCGTAATACAGCTCAA

-3641 CCTGACTTTTCCACCCTCAGCCTCCTTGATTCTCTAAATGAAGCCTGTCCACAATTGAAGCTCCT

-3576 TGTCTTTGCTCCTGCAAATTTGTTCAATCTCCTGGCTGTGTTGTGCTGGTCTCTGTCTATCTAG

FIGURE 14D

-3511 AGCTGTGGATATCATGGTATCTATTGTCTATCATGCTAGCCATGAACCACATGTGGCTGGTGAGC
-3446 ATTTTATATGGTACTAGTCTAAATTGACATCTACTGTGAGTGTAAAAATGTGCATTATGTTTTGA
-3381 AGACTGTACACAAAATTTAATTATCTCATGAATAATTTTAGATTGGTTATATGTTGAAATTATAA
-3316 TATTTTGGATATACTATGCTAAATAAAACATATTATTAAAAATTAACCTCACCTGTTTCTTTTCCT
-3251 CTTTCAATATGGCTACTAGAGCTTTTTAAATTGCATTATGTGACTTTATTGGACAGTACCGATTG
-3186 AATGCCCTCAACCACATCACCTCACCACAGCCACCTCTACCTGTAGTGATCATACCACTTCTTTA
-3121 GGCACACTGCCTGCATTAAAGGGCAATGAATGCCCTTTTCATCTTCTCCACTAGATGTAGTTTCTTT
-3056 TTTCTTTGAGAGCCATCATCACCATCATGGTTGACACCATGAACCTATCTGAAGATGTCAGCCAT
-2991 AGACTGCTTGATATTCTACAGGAAAGATCAGATTTTAAAGTGAATCTACCCATGTTATTAGCAG
-2926 TGTGTATCTTTCACACATTACACAGCCTCTCTAAGCCTCATTCTCTCTCTGTAAGATGGGGAT
-2861 GATAATAACCCATCTCAAATGTTTACTATGAGGATTATTCAAAGAATGGCAAATAGCAAGTGCTT
-2796 AATAAATGATAACTAGTACTACCGCCACTACTGTTGTTTTATTGTATTAGATTATGAACTCTCT
-2731 AAGGACCATTTCGGATGGAGGATAAGAGACCATTGATGTGGGCAGTGATGAGGCCTTCTGTTG
-2666 CACCTGGAAAGGTCAACTATATACAAGCCTGCAAGTCATTCTATAGGAGCAGGCCCCAGTGACCA
-2601 GACTCTATAGACTGTCTCCTCTTTCTGAGAGGGACAGCCATCTCTAGGTTGACTAACCTCTGAA
-2536 GCTCCTTGCAATGGCTTTTGTGCTATGAGCCATGGATGATTCCAGACTAATCCGAGAAATGCTCGT
-2471 CAAAACCCCAAGGAATTACTCAAATACTGACATAACAGACATTTTGTAGTGGAAGAGCCGAGTTT
-2406 TTTTAAATATTCTGAAACTCATTGTTTTTAAATGCATGAGATGGCCAAGGTCTTGCTAAGAGCT
-2341 GGCTGCAAAGCGAAAGGCAGAGAGAATGAAACCCATAGAGAGGCAGAATAACCAGAAAGGTTGG
-2276 GACTCGTTTATTTTATAATGTAAATTAGTCTATTATGAAACAATACTTGTTTACTGGTGAAAAAT

FIGURE 14E

-2211 TGGAAAATACAAAGAATAAAAGGAGGAAAAAATCACTCTTTAGTTTCACAAGCCAAATCAAGCC
-2146 ACTATTAAATGGTGGTTTACTTCCTTTTATTAATTTTCTGTACATATTTTGCATAATCATGTT
-2081 GTATGTACAATTTTATGTTCATTTTTCAATATTAACGGTGTCTTTCAAATTTCTTAATGACAA
-2016 AAATAATATATGCTCATAATAGAACATTTTAAATGCAAATAAAACAAATAAATGTTAAAATTTA
-1951 GTAATATTTATTAAATTTTCTCCAAGTGCAGGAAATTACAAATGTAACAACCTAATTCCTAGTG
-1886 GCCTAATAACCTATTTCCAGACCTCTTCTCATTACAAGGAAAACTCATATGCAGATAGTTCTA
-1821 AAGGTATGAAGTGAAAAGATAAAGATTTTCTTCCTTGCTGCATCCTCACCCTATCAGCATTATT
-1756 CCCCAGGGTAACTACTATTAAATAGATAGTAATTCTACCCAAAGGAAAAATCATATGCATATAAC
-1691 AGCATCATATGTATACCTTTCTAGTAACTTACAAAACAAATGATAATATCATATCCTTTCTTATG
-1626 TGTATTGCTCTTTTCACTAAATGTATCTGTGATATGTGTCTATATCAGCTGATTGTCCTTTTGA
PvuII (-1580)
-1561 TGGCTGAATAATATTCCATCTTGTCACGIGATAGTATTACTTGACAAGCTCCCTGCTGATGGAC
-1496 ATTTGTCTTTGTTACTATGATAGTAATATAATCAACATTTATATATGTTTTGTATGTATCTATAA
-1431 TACACATGCACATACACATGCATATTTCTGCAGGGATAGCCATAGTAAATAACTAGTAACGGTAT
-1366 TGCAAGTTAAAGGAACAATCTCATTGCTTGAAATTTTAAATTTTGAAATACACTGCCAATTTTCA
-1301 TGGTCTCTCCTTGTAAGCTAGTTTGGGCTTTCTCACAGCATGACAGGCTCAGGGCAGTCAGACCA
-1236 TCCTGGCCAAAGAGCAGAGTGCCACAGACCACAACTGCTTCTAATCAGCCATCTTCCCAAAGCCT
-1171 TCTCTTTTTTCTATTAAATAACTTTGTATGAGATTCCATCTTAATACTTTTCTGTGTTTGGTCTT
-1106 GTAAGAGCTTATTTTCTGAACCAGGAAGTGGTTCAGGGCGGTTTTTCTAACTTCACAGAGCTCC
-1041 CTCTTCGTAGCTTTTGTGAAATGGTCAAAAACATAGCAGCCTGCCTTCTGAGTTCTCCATCCC
-976 ACCCTGGTTGGGCCTTCTCTATCCTTGCTGTGTTGTTTATATCCTGCTGAAGTGTGATTCCACT

FIGURE 14F

-911 TGTGCAGTTTCTCCTCTGTGTAGGATCAAAAGGGCTGTGGCTGGTTGGTTTGAAAATTTCTTATAC
-845 CCTAGACTATTCCAGTGCCTTTTCAGAAAGTTTCCAAGGCCCTCTCACACTAATCTATTATCATATTG
-779 GGCAAAACTCCTTGCAGTTTCAGCTACTATTCCCTGATTGACTTTTCAGTAAATCTATCTCTCAGT
-713 CTTTCAGTATCCAAAGAAGATTGGTTCTAGGACCACCATCCCGCTGCCTCCACAGATACCAAATC
-647 AGAGGATGCTCAATTCCCTCTTATAAAACGTTGCAGTATTTGCAATATAATCTGCACATGTATTTCT
-581 GTATATTTTAAATCATCCCTAGATTACTTATAATACCTGATACAATATAAATGCTAAATAGCTGTA
-515 ACACTGTATCTTTAAAATTTACATTATTTTTTGTGTGTATTATTATTTTTATTGTATTTTTAAA
-449 AAATATTTTCCATCTACAGTCAGTAGAATCCACGGATACAGAACCTATGGATAGGAAGGACCAACT
-383 GTATCTTTTAGTGTTTTGAGGTTCTTG

FIGURE 14G

28/30

-356 AATTCTCAGGTGCTTTGCTTTCCCTTTGCTTTCTCCCAAGTCTGTGTTTTACAATTGCTTTAGTCA
-291 TTCACTGAAACTTTAAAAAACATTAGAAAACCTCACAGTTGTAAATCTTTTTCCCTATTATATA
-226 TATCATAAGATAGGAGCTTAAATAAAGAGTTTAGAACTACTAAAATGTAAATGACATAGGAAA
-161 ACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGAGGACCATCTCATATAAATA

CAP (-81)

-96 GGCCATACCCACGGAGAAAGGACATTCTAACTGCAACCTTACGAAGCCTTTGCTCTGGCACAACA

AUG (1)

-31 GGTAGTAGGGGACACTGTTCGTGTGTGCAAC ATG ACC AAC AAG TGT CTC CTC CAA
25 ATT GCT CTC CTG TTG TGC TTC TCC ACT ACA GCT CTT TCC ATG AGC TAC
73 AAC TTG CTT GGA TTC CTA CAA AGA AGC AGC AAT TTT CAG TGT CAG AAG
121 CTC CTG TGG CAA TTG AAT GGG AGG CTT GAA TAC TGC CTC AAG GAC AGG

PvuII (199)

169 ATG AAC TTT GAC ATC OCT GAG GAG ATT AAG CAG CTG CAG CAG TTC CAG
217 AAG GAG GAC GCC GCA TTG ACC ATC TAT GAG ATG CTC CAG AAC ATC TTT
265 GCT ATT TTC AGA CAA GAT TCA TCT AGC ACT GGC TGG AAT GAG ACT ATT
313 GTT GAG AAC CTC CTG GCT AAT GTC TAT CAT CAG ATA AAC CAT CTG AAG
361 ACA GTC CTG GAA GAA AAA CTG GAG AAA GAA GAT TTC ACC AGG GGA AAA
409 CTC ATG AGC AGT CTG CAC CTG AAA AGA TAT TAT GGG AGG ATT CTG CAT
457 TAC CTG AAG GCC AAG GAG TAC AGT CAC TGT GCC TGG ACC ATA GTC AGA
505 GTG GAA ATC CTA AGG AAC TTT TAC TTC ATT AAC AGA CTT ACA GGT TAC

BglII (565)

553 CTC CGA AAC TGAAGATCTCCTAGCCCTGTGCCTCTGGGACTGGACAATGTGCTTCAAGCATCT
615 TCAACCAGCAGATGCTGTTTAAGTGACTGATGGCTAATGTACTGCATATGAAAGGACACTAGAAG

FIGURE 15A

680 ATTTTGAAATTTTTATTAATTATGAGTTATTTTATTTTAAATTTTATTTTGGAAAATAAA
745 TTATTTTGGTGCAAAAGTCAACATGGCAGTTTAAATTCGATTGATTATATAACCATCCATA
810 TTATAAAATTGCCAAGTACCTATTAGTTGTTCTTTTAAATATACCTGCAAAGTAGTATACTTT
875 CTGGCCCCCTGCCTTTAAGGAATTTAAATTCAGAAAGCCATGATGGAATATATAAGGTAAGAGA
940 CAATAAGGGGACCTGAACCTTATGGGGGAATAAATATGGCATGAACTGCTGTGGGATTAAAGAG
1005 AAAAGGAAAGCTGGAGGGTCTGGAATAAACCTGGGGTTCCCATTCCTCCTACTGTGTGTTCCAG
Ball (1099)
1070 ATTCTCTCATCATAAAGTTAGAATTGAGCTGCCCATCAGGAATAGCCAGAGGAATATGTCAGCTT
1135 TTGTGTTCTCCCTAACCTTCCCCAGTTATTTGGGGGATCACTTTGCTCCTCGAAAGATTTTAAA
1200 TAATTATGTGCCCCCACCATCCCTGCAA

FIGURE 15B

30/30

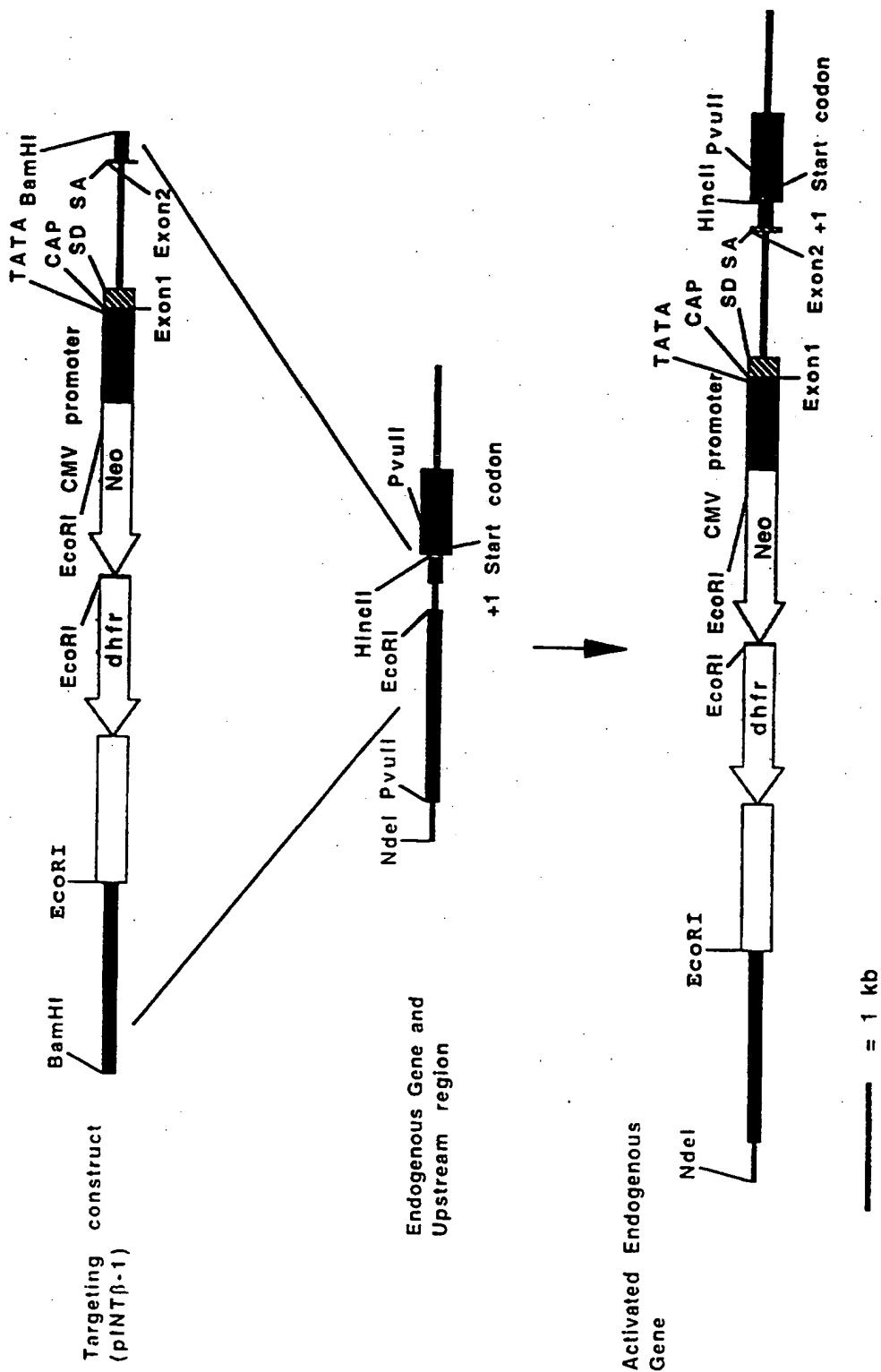


FIGURE 16

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/03377

| | | |
|---|--|--|
| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/19 C12N15/22 C12N15/55 C12N15/67 C12N9/16 C12N5/10 C07K14/52 C07K14/565 A61K31/70 A61K38/19 A61K38/21 A61K38/46 A61K48/00 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | WO,A,94 12650 (TRANSKARYOTIC THERAPIES INC) 9 June 1994 | 1 |
| Y | cited in the application see the whole document --- -/-- | 2-6, 13-18, 22-26, 33-38, 42-46, 51-55 |
| <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div> | | |
| <div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*&* document member of the same patent family</p> </div> </div> | | |
| Date of the actual completion of the international search <div style="text-align: center; font-weight: bold;">2 August 1996</div> | | Date of mailing of the international search report <div style="text-align: center; font-weight: bold;">07. 08. 96</div> |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016 | | Authorized officer <div style="text-align: center; font-weight: bold;">Hornig, H</div> |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/03377

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/03377

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/03377

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21, 41, 58 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/03377

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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